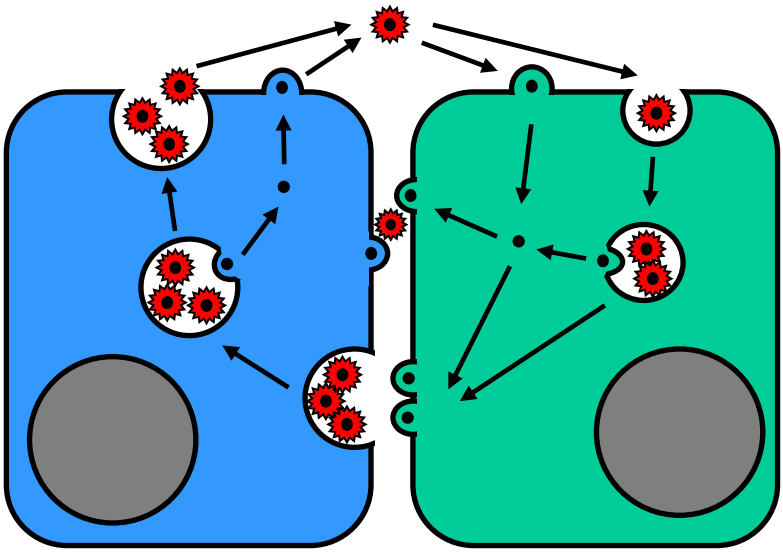
Universitat Autònoma de Barcelona
Facultat de Medicina
Departament de Biologia Cel·lular, Fisiologia i Immunologia

Characterization of the infection mechanism during cell-to-cell transmission of HIV-1



Marc Permanyer Bosser

2013

Doctoral Thesis

Universitat Autònoma de Barcelona

Facultat de Medicina, Departament de Biologia Cel·lular, Fisiologia i Immunologia

Characterization of the infection mechanism during cell-to-cell transmission of HIV-1

Marc Permanyer Bosser

Institut de Recerca de la SIDA (IrsiCaixa)

Hospital Universitari Germans Trias i Pujol

Doctoral Thesis UAB 2013

Thesis Director: **Dr. José A. Esté**

Tutor: **Dr. Dolores Jaraquemada**

El doctor **José A. Esté**, investigador principal de l'Institut de Recerca de la Sida (IrsiCaixa) de l'Hospital Germans Trias i Pujol de Badalona,

Certifica:

Que el treball experimental realitzat i la redacció de la memòria de la Tesi Doctoral titulada "*Characterization of the infection mechanism during cell-to-cell transmission of HIV-1"* han estat realitzats per en Marc Permanyer Bosser sota la seva direcció i considera que és apte per a ser presentat per a optar al grau de Doctor en Immunologia per la Universitat Autònoma de Barcelona.

I per tal que en quedi constància, signa aquest document a Badalona, 25 de Setembre del 2013.

Dr. José A. Esté

La doctora **Dolores Jaraquemada**, professora del Departament de Biologia Cel.lular, Fisiologia i Immunologia de la Unitat d'Immunologia de la Universitat Autònoma de Barcelona,

Certifica:

Que el treball experimental realitzat i la redacció de la memòria de la Tesi Doctoral titulada "*Characterization of the infection mechanism during cell-to-cell transmission of HIV-1"* han estat realitzats per en Marc Permanyer Bosser sota la seva direcció i considera que és apte per a ser presentat per a optar al grau de Doctor en Immunologia per la Universitat Autònoma de Barcelona.

I per tal que en quedi constància, signa aquest document a Barcelona, 25 de Setembre del 2013.

Dr. Dolores Jaraquemada

A la meva família

SUMMARY

Cell-to-cell HIV-1 transmission is a highly efficient mechanism of virus spread, and its relevance for *in vivo* dissemination in the active sites of replication, namely, primary and secondary lymphoid tissues, seems probable. Transmission of HIV antigens from infected to uninfected CD4+ T cells occurs through cell-to-cell contacts requiring exclusively the HIV envelope protein gp120 and the CD4 receptor interaction to induce the endocytic uptake of viral particles into trypsin-resistant compartments in the target cell. However the fate of transferred HIV antigens and whether internalization of HIV particles through an endocytic pathway can initiate a productive infection have not been completely understood. In the present work we have found that IgGb12, an inhibitor of virus attachment to CD4, prevented the infection of HIV-loaded target cells suggesting that endocytosed viral particles required resurfacing and reaching the extracellular environment to engage CD4 receptor to initiate a productive infection. Previous confocal microscopy studies found that clathrin and dynamin proteins colocalized with HIV particles in the target cell. However dynasore, a dynamin-dependent endosomal scission inhibitor did not prevent virus capture, virus-cell fusion or virus replication after cell-to-cell transfer of HIV particles into primary CD4+ T cells suggesting that endosomal maturation was not required for any step of the HIV infection cycle. Moreover, quantification of total viral DNA production indicated that all anti-HIV agents blocked cell-free and cell-to-cell virus transmission with similar potency discarding that cell-to-cell transmission could contribute to the persistence of the virus during antiretroviral therapy. Finally, it was observed that cell-to-cell transfer of HIV-1 antigens was dependent on the degree of actin polymerization of target primary CD4+ T cells. Thus, phenotypic differences in the cortical actin between naïve and memory CD4+ T cell subsets determined the efficiency of viral antigen transfer inducing distinct susceptibilities to HIV-1 infection. Our results reinforce the idea that endocytosed virus after cell-to-cell contacts may represent an itinerant virus reservoir able to induce the trans-infection of bystandard T cells, but not leading to effective virus replication from within internal endosomal compartments.

RESUM

La transmissió cèl·lula a cèl·lula del VIH-1 és un mecanisme altament eficient de disseminació viral, i la seva rellevància durant la difusió *in vivo* en els llocs actius de replicació, és a dir, en els teixits limfoides primaris i secundaris, sembla probable. La transmissió d'antígens del VIH de cèl·lules infectades a cèl·lules T CD4+ no infectades es produeix a través de contactes cèl·lula a cèl·lula que requereixen exclusivament de la interacció entre la proteïna de l'embolcall del VIH gp120 i el receptor CD4 per induir la captació endocítica de partícules virals en compartiments resistents a la tripsina en la cèl·lula diana. No obstant això, no ha estat completament estudiat el destí dels antígens del VIH prèviament transferits i si la internalització de partícules del VIH mitjançant endocitosi pot donar lloc a una infecció productiva. En aquest treball es mostra com IgGb12, un inhibidor de la unió del virus a CD4, va impedir la infecció de les cèl·lules diana carregades amb el VIH suggerint que les partícules virals endocitades van necessitar tornar a la superfície i arribar novament l'espai extracel·lular per interaccionar amb el receptor CD4 i iniciar una infecció productiva. Estudis previs realitzats amb microscòpia confocal van trobar que les proteïnes clatrina i dinamina colocalitzaven amb les partícules del VIH a la cèl·lula diana. Tot i això, dynasore, un inhibidor de la escissió endosomal no va impedir ni la captura de virus, ni la fusió del virus amb la cèl·lula ni la replicació del virus després de la transferència cèl·lula a cèl·lula de partícules del VIH suggerint que la maduració endosomal no és necessària per cap etapa del cicle d'infecció del VIH. D'altra banda, la quantificació de la producció de DNA total va indicar que tots els agents anti-VIH inhibien la transmissió de virus lliure o cèl·lula a cèl·lula amb una potència similar descartant que la transmissió cèl·lula a cèl·lula pogués contribuir a la persistència del virus durant la teràpia antiretroviral. Finalment, es va observar que la transferència cèl·lula a cèl·lula d'antígens del VIH-1 era depenent del grau de polimerització de la actina de les cèl·lules diana CD4+ T. D'aquesta manera, les diferències fenotípiques en l'actina cortical entre les cèl·lules CD4+ T naive i memòria van determinar la eficiència de la transferència d'antígens virals induint diferents susceptibilitats a la infecció per VIH-1. Els nostres resultats reforcen la idea de que, després dels contactes cèl·lula a cèl·lula, els virus endocitats poden representar un reservori de partícules virals itinerant capaç d'induir la trans-infecció de les cèl·lules adjacents, però no poden induir una replicació eficient a partir dels compartiments endosomals.

RESUMEN

La transmisión de célula a célula del VIH-1 es un mecanismo altamente eficiente de diseminación viral, y su relevancia durante la difusión *in vivo* en los sitios activos de replicación, es decir, en los tejidos linfoides primarios y secundarios, parece probable. La transmisión de antígenos del VIH de células infectadas a células T CD4+ no infectadas se produce a través de contactos célula a célula que requieren exclusivamente de la interacción entre la proteína de la envuelta del VIH gp120 y el receptor CD4 para inducir la captación endocítica de partículas virales en compartimientos resistentes a la tripsina en la célula diana. Sin embargo, no ha sido completamente estudiado el destino de los antígenos del VIH previamente transferidos y si la internalización de partículas del VIH mediante endocitosis puede dar lugar a una infección productiva. En este trabajo se muestra como IgGb12, un inhibidor de la unión del virus a CD4, impidió la infección de las células diana cargadas con el VIH sugiriendo que las partículas virales endocitadas necesitaron volver a la superficie y alcanzar nuevamente el espacio extracelular para interaccionar con el receptor CD4 e iniciar una infección productiva. Estudios previos realizados con microscopia confocal encontraron que las proteínas clatrina y dinamina colocalizaban con las partículas del VIH en la célula diana. A pesar de esto, dynasore, un inhibidor de la escisión endosomal no impidió ni la captura de virus, ni la fusión del virus con la célula ni la replicación del virus después de la transferencia célula a célula de partículas del VIH sugiriendo que la maduración endosomal no es necesaria para ninguna etapa del ciclo de infección del VIH. Por otra parte, la cuantificación de la producción de DNA total indicó que todos los agentes anti-VIH inhibían la transmisión de virus libre o célula a célula con una potencia similar descartando que la transmisión célula a célula pudiera contribuir a la persistencia del virus durante la terapia antiretroviral. Finalmente, se observó que la transferencia célula a célula de antígenos del VIH-1 era dependiente del grado de polimerización de la actina de las células diana CD4+ T. De esta manera, las diferencias fenotípicas en la actina cortical entre las células CD4+ T naive y memoria determinaron la eficiencia de la transferencia de antígenos virales induciendo distintas susceptibilidades a la infección por VIH-1. Nuestros resultados refuerzan la idea de que, después de los contactos célula a célula, los virus endocitados pueden representar un reservorio de partículas virales itinerante capaz de inducir la trans-infección de las células colindantes, pero no pueden inducir una replicación eficiente a partir de los compartimientos endosomales.

ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
CAp24	HIV antigen capsid p24
CDC	Center for Disease Control
CMFDA	CellTracker™ Green, 5-chloromethyl fluorescein diacetat
CCR5	Chemokine receptor 5, also known as CD195
CXCR4	CXC Chemokine receptor 4, also known as CD184
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DDAO	CellTrace™ Far Red, Dichloro-DimethylAcridin-One
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
EC50	50% effective concentration
EEA1	Early endsosomal antigen 1
ELISA	Enzyme-lynked immunoSorbent assay
Env	HIV envelope glycoprotein
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GALT	Gut-associated lymphoid tissue
GFP	Green fluorescence protein
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HTLV	Human T-cell leukaemia virus
ICAM	Intercellular adhesion molecule
IL-2	Interleukin-2
IN	HIV integrase enzyme
IS	Immunological synapse
Lamp1	Lysosomal-associated membrane protein 1
LAV	Lymphadenopathy associated virus
LFA-1	Lymphocyte function-associated antigen 1

LTR	Long terminal repeat
MA	HIV matrix protein (p17)
mAbs	Monoclonal antibody
MFI	Mean fluorescence intensity
MOI	Multiplicity of infection
MTOC	Microtubule organizing center
mRNA	Messenger RNA
NC	HIV nucleocapsid (p7)
NIH	National Institutes of Health
NMab	Neutralizing monoclonal antibodies
NRTI	Nucleoside reverse transcriptase inhibitor
PBMCs	Peripheral Blood Mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohaemmagglutinin
PMA	Phorbol 12-myristate 13-acetate
Pol	HIV polymerase enzyme
PR	HIV protease enzyme
qPCR	Quantitative real-time PCR
Rev	Regulator of virion protein expression
RNA	Ribonucleic acid
RRE	Rev response element
RT	HIV reverse transcriptase enzyme
SSC	Side Scatter
VS	Virological synapse
VSV	Vesicular stomatitis virus
WT	Wild type

TABLE OF CONTENTS

INTRODUCTION

1.- History

In June 1981, the Center for Disease Control and Prevention (CDC) described what is considered the first reported cases of AIDS [6]. In 1983, only two years later, its etiological agent was firstly identified [7] and then confirmed [8, 9] as a virus belonging to the general family of T-lymphotropic retroviruses and as the causative agent of several pathological syndromes affecting the normal cellular immune function including AIDS [7]. In 1986, the International Committee on the Taxonomy of Viruses designated the previously named LAV or HTLV-III as human immunodeficiency virus (HIV) [10]. More than twenty years have passed since the first 5 reported cases in Los Angeles. According to the UNAIDS Reports on the global AIDS epidemic 2012, 34 million people was leaving with HIV at the end of 2011 making AIDS one of the most serious health challenges of the world [11].

2.- Immunopathogenesis of HIV-1 infection

Most of the HIV-1 infections occur by sexual exposure through the genital tract or rectal mucosa (**Figure 1**). It is thought that viruses cross the mucosal epithelium by transcytosis or by making direct contact with underlying mucosal Langerhans cells and CD4+ T cells [12-14]. After being transported across the epithelium, HIV reaches its main target for infection in the genital mucosa which is CD4+ memory T cells. During this initial phase of infection, viral RNA is undetectable in the plasma. After approximately 10 days, HIV reaches lymph nodes, where the infection is strongly amplified due to the high local density of CD4+ T cells. At the same time, DCs begin to present processed HIV antigens to naïve B cells and T cells, thereby initiating the adaptive response to the infection. Next, the virus replicates rapidly and spreads throughout the body and to other lymphoid tissues, particularly in the gut-associated lymphoid tissue (GALT), where activated CD4+ memory T cells are present in high numbers. During this period, the plasma viraemia increases exponentially to reach a peak, usually more than a million of RNA copies per ml of blood. After the peak viraemia a balance between the virus turnover and the immune responses is established. Persistent HIV-1 infection would lead to chronic HIV-associated immune activation until depletion of CD4+ T cell reservoirs that could not be replenished leading to exhaustion of the immune system and development of acquired immune deficiency syndrome (AIDS).

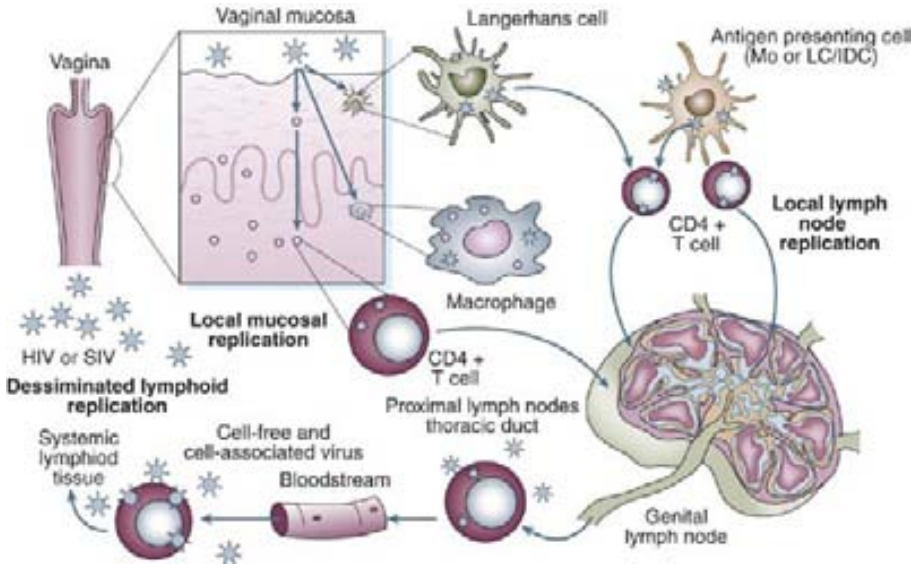


Figure 1. Dissemination of HIV-1. HIV crosses the mucosal epithelium by trancytosis or by making direct contact with intraepithelial dendritic cells and CD4+ T cells. After mucosal infection HIV-infected cells migrate to proximal lymph nodes were infection is rapidly amplified and disseminated through the lymphatic system to the blood and then to all lymphoid tissues. [2]

3.- The replication cycle of HIV-1

As an obligatory intracellular parasite HIV-1 can only replicate inside human cells. The steps of the HIV life-cycle are described below (**Figure 2**):

3.1.- HIV Viral Entry

The principal targets for HIV-1 infection are T cells, and to a lesser extent macrophages and dendritic cells. This tropism is determined at the level of viral entry by the use of CD4 as the primary receptor and the use of one of the two co-receptors that define two different viral strains. R5 strains of HIV use CCR5 as their co-receptor and can, therefore, enter macrophages, DCs and T cells, whereas X4 strains of HIV use CXCR4 as a co-receptor and can infect only T cells. The engagement of the HIV envelope glycoprotein (Env) with cell receptors triggers conformational changes that culminate in viral and host cell membrane fusion and release of the viral core into the cytoplasm. Inhibitors of the different steps of HIV-1 entry into target cells have been identified including attachment inhibitors such as the mAb IgGb12, coreceptor antagonists such as Maraviroc or AMD3100 for CCR5- and CXCR4-using viruses respectively and fusion inhibitors such as enfuvirtide (T-20) [15-17].

3.2.- HIV uncoating, reverse transcription, nuclear import and integration

Once internalized, HIV is uncoated, and its single-stranded RNA is retro-transcribed by the RT into a double-stranded DNA. Reverse transcription can be inhibited by nucleoside and non-nucleoside RT inhibitors (NRTIs and NNRTIs, respectively). NRTIs such as Zidovudine (AZT) or Tenofovir (TDF) mimic natural dNTPs and are incorporated into the viral DNA by the RT.

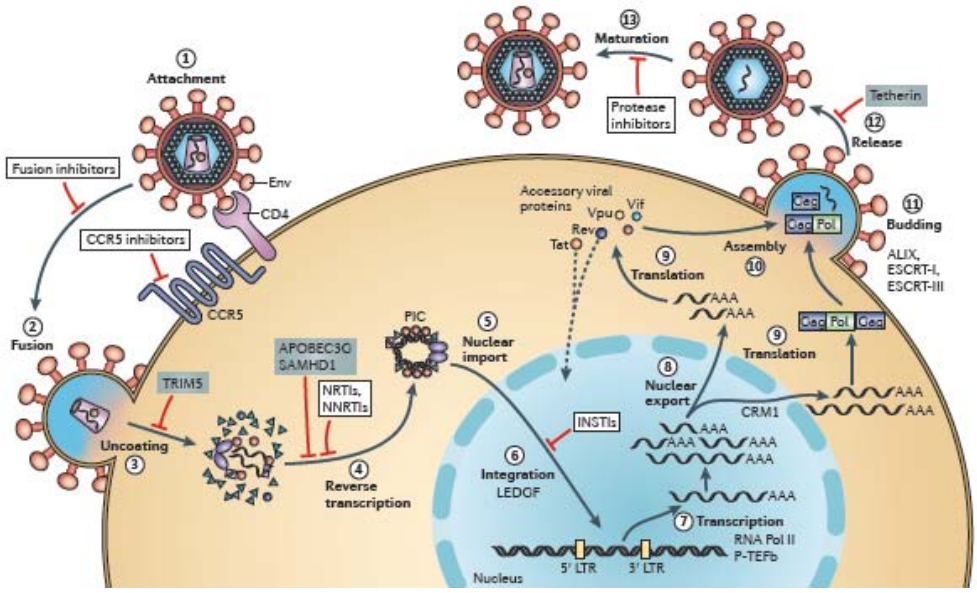


Figure 2. The HIV-1 life cycle. After binding to CD4 and one coreceptor (CCR5 or CXCR4), viral fusion with the cell membrane results in entry of the viral core into the cytoplasm. Following reverse transcription, the viral cDNA is transported to the nucleus to form the integrated provirus. Genomic viral transcripts exported from the nucleus and newly transduced viral proteins are packaged to form new virions. After budding, particle maturation occurs by protease cleavage. [1]

NNRTIs binds at different site on the RT and inhibit its movement. Then the viral genome, associated with Vpr, MA and host proteins enters the nucleus. Finally, integration of HIV into the host genome is catalysed by IN generating the integrated form of HIV called provirus.

3.3.- HIV transcription and translation

The transcriptional activity is dependent on cellular factors including the host cell RNA polymerase II machinery but also viral factors. The small mRNAs produced during the early transcription phase are directly exported to the cytoplasm and encode for the regulatory proteins Nef, Tat and Rev. Regulator of the viral gene expression (Rev) acts

as an adaptor protein which binds to the Rev response element (RRE) and mediates cytosolic export of unspliced and singly spliced mRNAs. The viral transactivator protein (Tat) binds newly transcribing mRNAs and by promoting recruitment of other cellular factors stimulates transcription elongation. Negative effector (Nef) facilitates viral assembly. Viral structural and enzymatic proteins are synthetized in the cytoplasm and transported to the plasma membrane.

3.4.- HIV assembly, budding and maturation

The formation of new HIV virions occurs at the plasma membrane by packaging two copies of genomic viral RNA, the viral envelope (Env) protein, the Gag polyprotein, and the three viral enzymes: protease (PR), reverse transcriptase (RT), and integrase (IN). Conversion of the immature virus in its mature infectious form requires the activation of PR which cleaves Gag precursor releasing three structural proteins: matrix (MA), Capsid (CA) and Nucleocapsid (NC).

4.- Mechanisms of HIV-1 dissemination

4.1.- Cell-free and cell-to-cell spread

HIV-1 can propagate mainly via cell-free viral transmission or via cell-cell contacts (**Figure 3**). Cell-free viral transmission is considered the "classical" route of viral infection which occurs after binding of cell-free virions to a permissive host cell via receptor interactions followed by fusion and entry into the host cell cytoplasm. Further, dendritic cells can capture HIV-1 virions and without being themselves productively infected, re-present infectious viruses to permissive target cells (a mechanism known as trans-infection). Finally, HIV-1 can propagate directly from infected to non-infected T cells through direct transmission of viral antigens. Cell-to-cell viral transmission has been shown to be more efficient than cell-free virus spread [18-20] and consequently to promote higher multiplicity of infection [21, 22]. While the oncogenic retrovirus human T cell leukaemia virus-type 1 (HTLV-I) uses this mechanism as a primary dissemination pathway between cells and between individuals [23], the relative contribution of cell-to-cell and cell-free virus transmission during *in vivo* viral dissemination has not been completely clarified [24].

4.2.- Cell-to-cell transmission of HIV-1

4.2.1.- History

Cell-to-cell spread, that is, the directed movement of viral particles between cells has been adopted as a transmission mechanism by different families of viruses (Herpesviruses, Poxviruses, Paramyxoviruses and Retroviruses) [3]. Specifically for retrovirus, early evidences came from studies in the field of immunological antigen presentation which revealed the molecular mechanisms of cell-cell adhesion and the intercellular communication between cells [25, 26]. However, was Steinman who first described the transfer of HIV particles between Dendritic cells and T cells through the formation of cell-cell conjugates [27, 28]. Consistent with these early reports, later studies demonstrated that cell-to-cell contacts facilitated the transmission of HIV antigens by concentrating virus, receptors and coreceptors during the formation of the "infectious synapse" between dendritic cells and T cells [29] or between T cells [30]. More recently, several articles have confirmed and extended these observations even using different cell types including dendritic cells, T cells or macrophages [19, 31-34]. Latest articles have pointed out that after cell-to-cell transfer viral antigens were internalized through an endocytic pathway [35] in a coreceptor-independent manner [36].

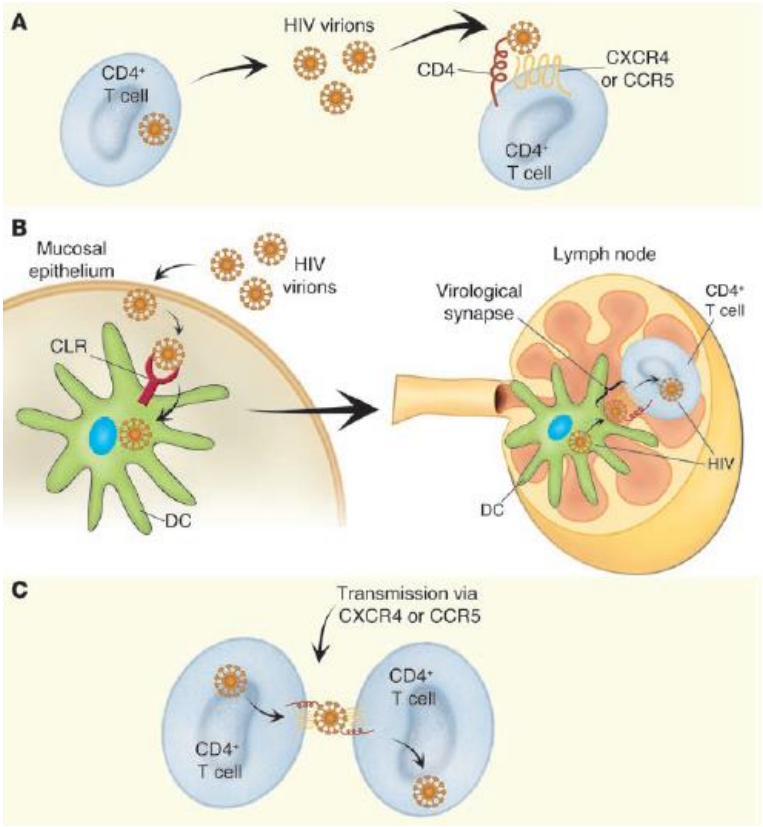


Figure 3. Mechanisms of HIV propagation. A. Cell-free viral transmission. Cell free virions bind to the target cell, followed by viral fusion and subsequent viral replication. **B. DC-T cell viral transmission.** DC transfer captured HIV particles to target CD4+ T cells. **C. T cell-T cell viral transmission.** HIV is directly transmitted from an HIV-infected CD4+ T cell to second CD4+ T cell. [4]

4.2.2.- Cell-to-cell contacts: structure and organization

A central requirement of a synaptic structure is the formation of an adhesive contact between cells that provide sufficient stability for the antigen transfer. The cell-cell contact that allows viral transmission between cells has been called, by analogy with the immune synapse, virological synapse [4] (**Figure 4**). Thus, the virological synapse (VS) is defined as the cytoskeleton-dependent stable adhesive junction in which viruses are transmitted directly and efficiently from an infected (effector) cell to an uninfected permissive (target) cell [23, 30, 37, 38]. Viral

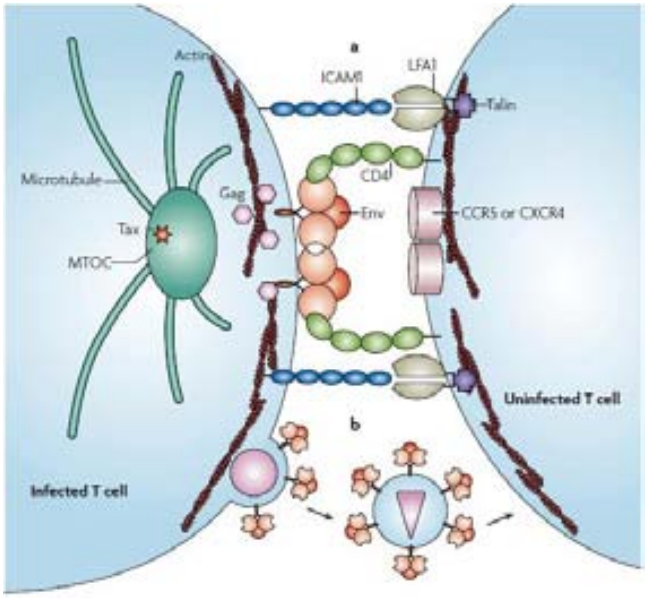


Figure 4. Structure of the virological synapse. HIV-1 envelope glycoproteins (Env) expressed on the infected cell plasma membrane interacts with the CD4 and CCR5 or CXCR4 receptors on the target cell. The adhesion molecules LFA-1 and ICAM-1 engage and stabilize the cellular conjugate. The actin cytoskeleton and the secretory apparatus (MTOC) are also polarized at the cell-cell contact zone. [3]

antigens presented by effector cells and cellular receptors presented by target cells are recruited at the interface of the cell-cell contact and thus viral antigens can be rapidly transmitted through the synaptic junction. The CD4 receptor engagement by the HIV Env glycoprotein gp120 triggers an actin-dependent recruitment of CD4, CXCR4 and the lymphocyte function-associated antigen 1 (LFA-1) on the target cell and Env-Gag coclustering in the effector cell [30]. Even though interactions between adhesion molecules LFA-1 and intercellular adhesion molecule 1 (ICAM-1) or ICAM-3 were involved during conjugate formation and cell-to-cell transfer of HIV-1 [38], the main driving force for cell-to-cell transfer of HIV antigens was the interaction between the HIV envelope protein gp120 and CD4 receptor [39]. Furthermore, efficient assembly, budding and VS formation requires actin and tubulin cytoskeleton components [37], elements of the regulated secretory pathway [40] and the maintenance of the lipid raft integrity since their disruption disperses the localization of Env within the plasma membrane and eliminates Gag clustering at the site of cell-cell contact [41]. Finally, other elements sheared with the T cell immunological synapse (IS) have also been shown to be required during the cell-to-cell HIV-1 spread [40, 42]. The ZAP-70 kinase, involved in the IS formation, was required for the correct reorientation of Gag protein and MTOC within the infected cell during VS formation [42]. Also HIV may hijack

other elements of the regulated secretory pathway such as CTLA-4 and Fas ligand to direct secretion of viral antigens at the VS and enhance its dissemination [40].

4.2.3.- Advantages to spread through cell-to-cell transmission

Cell-associated HIV transmission allows overcoming various biophysical, kinetic and immunological barriers [3].

- Infected cells can adhere and cross the mucosal epithelial barrier by transmigration that would otherwise be impermeable to free-viral particles [43, 44].
- Polarized and directed cell-to-cell transmission allows more rapid replication kinetics by eliminating the fluid-phase diffusion and by promoting a more efficient receptor engagement [30] and viral entry [22].
- Transmission of viral particles between cells may confer protection from innate and adaptive immune responses [19, 45, 46].

4.2.4.- Controversies between cell-free and cell-associated infections

4.2.4.1.- Susceptibility to inhibition

An important question arising from the higher efficiency of cell-to-cell transmission compared to cell-free virus spread [18-20] is related to whether both mechanisms are equally susceptible to inhibition. Besides, as previously demonstrated for viruses of other families including herpesviruses [47], poxviruses [48] and hepatitis C virus [49], the transfer of viral antigens by direct cell-cell spread could promote evasion from immune system or therapeutic interventions due to the impermeability of the tight junction formed between cells. Preliminary evidences suggested that cell-to-cell HIV-1 transmission was resistant to neutralizing monoclonal antibodies (NMab) or azidothymidine (AZT) [50]. Consistent with this result, later studies suggested that a polyclonal mixture of HIV-1 antibodies was less effective at blocking transfer of viral Gag [19] and viral fusion [46] when the virus was transferred between cells. Moreover, cell-to-cell transmission was shown to evade inhibition specifically by anti-gp120 CD4 binding site (CD4bs) directed inhibitors such as the mAb IgGb12 or the tetrameric fusion protein CD4-IgG2 but not by other entry or cell-directed inhibitors [45]. However, these results are in contrast to previous observations showing that virus attachment inhibitors including CD4-IgG2 [51] and other gp120 or gp41-directed

antibodies [52] effectively blocked cell-to-cell transmission. Consistent with these results, imaging analysis and time-of-addition studies were performed to demonstrate that a wide range of entry inhibitors could access preformed virological synapses and interfere with HIV-1 cell-to-cell infection suggesting that cell-associated HIV infection is sensitive to entry inhibition and is not an immune evasion mechanism [53]. However, a recent study have found cell-associated HIV infection less sensitive to the RT inhibitors tenofovir and efavirenz than cell-free HIV infection suggesting that cell-to-cell transmission represents a mechanism contributing to virus escape to the action of antiretrovirals and a mode of HIV persistence during antiretroviral therapy [5]. Thus, the question whether cell-free or cell-associated infections are equally susceptible to inhibition remains unclear. The fact that the effectiveness of the antiretrovirals could depend on the mode of HIV spread may have important consequences for the pathogenesis and the persistence of HIV infection

4.2.4.2.- The mechanism of HIV Entry: fusion or endocytosis

Another aspect that has led to conflicting results refers to the clathrin-mediated endocytosis as a mechanism of internalization of viral antigens and, to what extent, this mechanism leads to a productive infection of target cells. Previous reports have shown that after cell-to-cell transfer, HIV antigens accumulate into trypsin-resistant compartments within the target cell [19, 35, 36]. Moreover, antigen transfer only required HIV gp120 binding to CD4 receptor and occurred in the absence of membrane fusion or productive infection [36]. Further characterisation lead to the detection of transferred HIV-1 virions into compartments positive for clathrin, dynamin and the early endosomal marker EEA-1, suggesting that HIV particles were internalized through an endocytic pathway [35]. These results were confirmed using different microscopic techniques that allowed a detailed description of the cell-to-cell transfer of antigens [32]. However, Hübner et al. [32] did not resolve if transferred HIV particles finally reached the cytoplasm, if virus particles were able to uncoat and if that endocyted virus was able induce a productive infection. Following these line of evidences, single-particle imaging and virus population-based fusion assay were used to demonstrate that complete fusion occurred only in the endosome but not in the cell membrane suggesting the endocytic uptake as the mechanism leading to productive infection [54]. Similarly Dale et al. [46] showed that particle fusion required that the transferred virus undergo maturation within the cell endosome pointing a mechanism by which HIV could evade

antibody neutralization. The results showing endocytosis to be the mode of HIV entry are in apparent contrast to previous showing no overlap between HIV-1 antigen and early endosomes, and found no evidence of virus particles within endosomal structures in target cells exposed to HIV-1-infected cells [30, 53]. Also, Yu et al. [55] showed that in resting CD4 T cells, only the HIV envelope-mediated entry but not the VSV-mediated endocytosis can lead to viral DNA synthesis, suggesting alternative routes of viral genome delivery by endocytic and nonendocytic processes, but also highlighting differences in the mechanism of virus entry in transformed versus resting T cells.

4.3.- Other HIV-1 cell-to-cell transmission mechanisms

4.3.1.- DC-T cell trans-infection

DCs can capture and internalize viral particles in the absence of fusion events, transfer to interacting CD4+ T cells leading to productive infection and contributing to viral spread through a mechanism known as *trans*-infection [56]. Although initial studies identified the C-type lectin DC-SIGN as the HIV-1 binding factor on DCs, mediating *trans*-infection after viral capture through interacting with the HIV envelope gp120 [57, 58], later studies suggested other DC-SIGN-independent mechanisms [59-61]. More recently, Siglec-1 (CD169) has been identified as a general binding receptor that may capture viral particles and mediate *trans*-infection through interacting with sialyllactose-containing gangliosides exposed on viral membranes [62-64]. After viral capture, HIV-1 is internalized and transported into non-lysosomal, endocytic compartments [65]. Trans-infection occurs via the infectious synapse, a cell-cell contact zone that facilitates transmission of HIV-1 by locally concentrating virus and viral receptors [29].

4.3.2.- Trancytosis

Contact between HIV-1-infected cells and the mucosal pole of the epithelial cells promotes higher efficiency of trancytosis of infectious virus than cell-free HIV-1 particles suggesting an efficient mechanism for transmission of HIV across an intact epithelial barrier [43, 44]. Three epithelial molecules, namely, the heparin sulphate proteoglycan agrin as HIV-1 attachment receptor, beta-1 integrin and the previously described endocytic receptor for HIV-1 in epithelial cells galactosyl ceramide (GalCer) [44, 66], are required for virally mediated synapse formation, stabilization, and initiation of efficient HIV-1 endocytosis/trancytosis.

4.3.3.- Membranous intercellular connections

Besides the transmission mechanisms described above, it has been shown that HIV-1 can also spread through membranous intercellular unions formed between infected and non-infected cells called filopodia [67]or nanotubes [68]. Thus, viral particles would use these bridges to move between apparently distant cells. The main differences lie in the stability, the length and the speed of the virion movement. Nanotubes are 5-10 times longer and move 2-5 times faster than filopodial connections. Conversely, unlike filopodia, nanotube stabilization does not require HIV envelope gp120-CD4 receptor interactions [68].

5.- Susceptibility to HIV-1 infection of different CD4 T cell subsets

Naïve CD4+ T cells must interact with mature antigen-loaded dendritic cells to be successfully activated. This interaction takes place in the T-cell areas of secondary lymphoid organs leading to extensive T cell proliferation and differentiation into effector cells. Once the infection has been cleared most of the activated T cells die by apoptosis but a small percentage convert to memory CD4+ T cells [69]. Naïve and memory CD4+ T cells can be phenotypically differentiated by the expression of CD45RA and CD45R0 molecules, respectively. Moreover, naïve T cells exhibit a restricted pattern of migration, recirculating continuously between secondary lymphoid organs via blood and lymph and require long exposition to the related antigen to proliferate [70]. Conversely, memory T cells exhibit higher sensitivity to antigenic stimulation and express chemokine and adhesion receptors that enable them to enter into non-lymphoid tissues [69]. In infected patients, memory CD4+ T cells are preferentially infected and harbour more integrated viral DNA than naïve CD4+ T cells [71-73]. These findings are recapitulated by multiple *in vitro* studies showing that purified memory CD4+ T cells support higher levels of HIV-1 replication than naïve CD4+ T cells [72-75] but the mechanism underlying the different susceptibility to HIV-1 infection remains unclear.

Even though a subset of memory cells expressed higher levels of CCR5 and the level of CXCR4 was slightly higher on naïve T cells [76], the inherent resistance of naïve CD4+ T cells to HIV-1 infection may not be explained by the different expression of viral coreceptors [74, 76], the degree of cell activation [77] or induced by a host cell factor [77]. Importantly, the level of viral integration in naïve cells was lower than that in

memory cells [76] suggesting that restriction of infection occurs at the first steps of virus life cycle. Differences in both T cell subsets in the binding capacity [78], viral fusion [76] or viral DNA synthesis [77] could potentially explain the preference of HIV-1 for the memory T cell subset. However, differences in the susceptibility to infection in naïve and memory CD4+ T cell have not been evaluated during cell-to-cell transmission of HIV-1. Memory and naïve T cells differ in their migratory capacity which is mainly driven by cortical actin polymerization. Since cortical actin dynamics are required for the concentration of HIV antigens and its cellular receptors at the cell-cell contact zone [30] and for the cell-to-cell transfer of HIV antigens [19, 30, 32], we speculated that differences in the cortical actin between naïve and memory CD4+ T cells could affect their susceptibility to cell-to-cell transmission of HIV-1.

HYPOTHESIS & OBJECTIVES

Cellular contacts between HIV-infected donor cells and uninfected primary CD4+ T lymphocytes lead to virus transfer into endosomes. Moreover, recent evidences suggest that transferred HIV particles may fuse with endosomal membranes to initiate a productive infection. However, endocytic internalization occurs in the absence of virus fusion or replication. Thus, the role of endocytosis in the establishment of a productive infection and whether or not endocytic virus transfer represents an escape mechanism from the immune system or therapeutic agents remains highly controversial.

> **Objective 1:** To determine the infection mechanism of internalized HIV particles after HIV cell-to-cell transfer from infected lymphoid cells to uninfected primary CD4+ cells. This objective is addressed in *Chapter 1*.

Cell-associated infection disseminates HIV-1 more efficiently than cell-free virus infection. Recently, cell-to-cell transmission of HIV has been proposed as a mechanism contributing to virus escape to the action of antiretrovirals and consequently, a mode of HIV persistence during antiretroviral therapy. However, discrepant results have been obtained regarding the inhibitory efficiency of the antiretroviral compounds in both cell-free and cell-associated infection systems.

> **Objective 2:** To compare the inhibitory efficacy of nucleoside reverse transcriptase inhibitors in cell-free and cell-associated HIV infection. This objective is addressed in *Chapter 2*.

CD4+ memory T cells support higher levels of HIV-1 replication than naïve CD4+ T cells, but the mechanism underlying this different susceptibility remain unclear. Although, several studies have shown the viral dependence on the actin cytoskeleton during early and post-entry processes of infection, the role of the cytoskeleton during cell-to-cell transmission of HIV-1 into naïve and memory T cell subsets has not been well understood.

> **Objective 3:** To characterize the role of cortical actin and its contribution to cell-to-cell transmission of HIV-1 in naïve and memory T cell subsets. This objective is addressed in *Chapter 3*.

RESULTS

Chapter 1.- Trans-infection but not infection from within endosomal compartments after cell-to-cell HIV-1 transfer to CD4+ T cells

Marc Permanyer, Ester Ballana, Roger Badia, Bonaventura Clotet and José A. Esté

IrsiCaixa, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, 08916 Badalona, Spain.

J Biol Chem. 2012 Sep 14;287(38):32017-26.

Cellular contacts between HIV-1-infected donor cells and uninfected primary CD4+ T lymphocytes lead to virus transfer into endosomes. Recent evidence suggests that HIV particles may fuse with endosomal membranes to initiate a productive infection. To explore the role of endocytosis in the entry and replication of HIV, we evaluated the infectivity of transferred HIV particles in a cell-to-cell culture model of virus transmission. Endocytosed virus led to productive infection of cells, except when cells were cultured in the presence of the antigp120 mAb IgGb12, an agent that blocks virus attachment to CD4, suggesting that endocytosed virus was recycled to the outer cell surface. Confocal microscopy confirmed the colocalization of internalized virus antigen and the endosomal marker dynamin. Additionally, virus transfer, fusion, or productive infection was not blocked by dynasore, dynamin-dependent endosome-scission inhibitor, at subtoxic concentrations, suggesting that the early capture of virus into intracellular compartments did not depend on endosomal maturation. Our results suggest that endocytosis is not a mechanism of infection of primary CD4 T cells, but may serve as a reservoir capable of inducing trans-infection of cells after the release of HIV particles to the extracellular environment.

1.1.- Introduction

Viruses are obligatory intracellular parasites that take advantage of the host cell machinery to replicate and spread from infected to uninfected cells [3, 79, 80]. Cell-to-cell transmission has been shown to be a highly efficient mechanism of virus spread [5, 53], and its relevance for *in vivo* dissemination in the active sites of replication, namely, primary and secondary lymphoid tissues, seems probable. HIV may be transferred from infected to uninfected CD4+ cells [81, 82] by a mechanism that requires intimate cell-to-cell contacts involving the HIV envelope glycoprotein gp120 and the CD4 receptor but also accessory cell surface proteins [83]. Virus-cell fusion and initiation of a productive infection require engagement to CD4 and to one of the two alternative coreceptors, CCR5 or CXCR4. The various steps in the mechanism of virus entry are considered targets for anti-HIV intervention [15, 16].

Cell-to-cell transfer of HIV particles may be blocked by agents that prevent virus attachment, such as the anti-CD4 monoclonal antibody (mAb) Leu3a, the anti-gp120 mAb IgGb12, or the CD4-IgG2 fusion protein PRO542 [51], but is resistant to HIV entry inhibitors targeting virus coreceptors or gp41-dependent fusion [32, 82],

suggesting that virus attachment to CD4 is the sole factor necessary to induce the uptake of HIV particles [39] and that virus capture may occur in the absence of virus fusion and the initiation of a productive infection. Endocytic internalization and endosomal acidification have been shown not to be required to activate HIV entry into the cytoplasm [84-87].

Alternatively, several lines of evidence support clathrin-dependent endocytosis as an infectious pathway [19, 32, 36, 54, 88]. HIV fusion with endosomal membranes has been observed by electron microscopy [89]. Daecke *et al.* [90] proposed a role for endocytosis in productive entry of HIV-1 by using trans-dominant negative proteins that interfered with specific clathrin endocytic routes and effectively blocked virus replication. Complete fusion of HIV particles with HeLa cells has been observed to occur within endosome membranes [54], but complete fusion was blocked when endocytosis was inhibited [91]. Recent data suggest that after cell-to-cell transfer, virions first need to undergo maturation within endosomes, delaying membrane fusion and reducing sensitivity to patient antisera compared with cell-free virus [46]. Thus, the role of endocytosis in HIV replication and whether or not endocytic virus transfer represents an escape mechanism from the immune system or therapeutic agents remain highly controversial [5, 92].

Here, we show that primary CD4+ T lymphocytes take up virus particles into dynamin-containing compartments even in the presence of the endosome-scission inhibitor dynasore. Moreover, purified cells carrying endocytosed virus particles did not become productively infected if cultured in the presence of HIV attachment inhibitors such as the anti-gp120 mAb IgGb12, suggesting that endocytosed virus was recycled to the cell surface to initiate a productive virus infection.

1.2.- Materials and Methods

Cells. Peripheral blood mononuclear cells from healthy donors were purified by Ficoll-Hypaque sedimentation. CD4+ T lymphocytes were immediately purified (>95%) from peripheral blood mononuclear cells by negative selection using the CD4+ T cell enrichment kit (Stem Cell Technologies, Vancouver, Canada) and grown in RPMI 1640 L-glutamine medium (Invitrogen) supplemented with 10% (R10) heat-inactivated fetal calf serum (FCS; Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin.

When needed, CD4+ T cells were stimulated with phytohemagglutinin (PHA; Sigma) at 4 μg/ml and 6 units/ml interleukin 2 (IL-2; Roche Applied Science). MOLT-4 lymphoid cells (AIDS Reagent Program, National Institutes of Health, Bethesda, MD) were cultured in R10. Chronically HIV-1-infected MOLT cells were generated after the infection of MOLT cells with the NL4-3 X4 HIV-1 ($MOLT_{NL4-3}$). After the infection peak, the persistently infected culture was grown and characterized for Env expression and virus production. HEK293-T cells (AIDS Reagent Program) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Cocultures of Infected and Uninfected Cells. Nonstimulated primary CD4+ T cells (to minimize virus replication) were cocultured with uninfected or HIV-1 persistently infected $MOLT_{NL4-3}$ cells as previously described [36, 51, 88]. Purified CD4+ T cells were first labeled with the cell tracker CMFDA (Molecular Probes) and washed before being mixed with $MOLT_{NL4-3}$ cells. Briefly, 2.5×10^6 of both infected and target cells (1:1 ratio) were cocultured in 48-well culture plates in a final volume of 1 ml in the absence or presence of the following HIV-1 inhibitors: 80 nM neutralizing anti-gp120 mAb IgGb12 (Polymun Scientific, Wien, Austria); 4μM reverse transcriptase (RT) inhibitor 3-azido-3-deoxythymidine (AZT); 12.5 μM AMD3100 or 80 μM dynamin inhibitor dynasore (all from Sigma-Aldrich). Cocultures were incubated overnight at 37 °C. The capture of CAp24 antigen by primary CD4+ T cells was evaluated by flow cytometry as shown before (10, 11, 25, 27, 28). Prior to staining, cells were trypsinized to eliminate HIV-1 particles bound to the cell surface. For trypsin treatment, cells were washed with phosphate-buffered saline (PBS) and treated for 8 min at room temperature with 0.25% trypsin solution (Invitrogen). Trypsin was stopped by addition of FCS, and cells were then washed with PBS. For intracellular staining, cells were fixed, permeabilized (Fix&Perm; Caltag, Burlingame, CA), and stained with the anti HIV-CAp24 antigen mAb KC57 (Coulter). Cells were analysed in a LSRII flow cytometer (BD Bioscience) and identified by morphological parameters and CMFDA staining.

Isolation of Target CD4+ T Cells. CMFDA-loaded target CD4+ T cells were purified (>99% purity) from $MOLT_{NL4-3}$ cells by fluorescence-activated cell sorting (FACSAria II, BD Biosciences). After separation, contaminating $MOLT_{NL4-3}$ cells (<1%) were assessed by FSC/SSC parameters using flow cytometry. The possible contribution to infection of persistent $MOLT_{NL4-3}$ cells (<1%) was evaluated using the coculture

performed with the mAb IgGb12, a condition where HIV-1 uptake into CD4+ T cells is blocked, and therefore, infection of purified CD4+ T cells would only come from remaining $MOLT_{NL4-3}$ cells.

Culture of HIV-1-loaded cells. Isolated CD4+ T cells from each initial coculture condition were subdivided in three and cultured for 5 days in the following medium conditions: (i) 80 nM mAb IgGb12; (ii) 80 nM mAb IgGb12 and 4μM RT inhibitor AZT; or (iii) left untreated. CD4+ T cells were activated by adding 4 μg/ml PHA and 6 units/ml IL-2 to the medium. After 5 days, infection in target cells was assessed by enzyme-linked immunosorbent assay (ELISA) for HIV-CAp24 antigen detection in culture supernatants (Genscreen HIV-1 Ag EIA; BioRad Laboratories).

Determination of Anti-HIV Activity in Cell-free Virus Infections and Cell-Cell Transfer. The anti HIV activity using cell free virus infections was determined as described before [93]. Briefly, PHA-activated CD4+ T lymphocytes (1.5×10^5 cells/ well) were incubated with $HIV\text{-}1_{NL4-3}$ (200 $TCID_{50}/10^6$ cells) or mock-infected during 7 days at 37 °C, 5%CO2 in the presence of different concentrations of the corresponding test compound. HIV-1 CAp24 antigen production in the supernatant was measured by a commercial ELISA test as described above. To determine cytotoxicity, mock-infected cells were harvested and fixed with 1% formaldehyde. Cell death was quantified by flow cytometry in forward *versus* side scatter plots. Dead cells showed increased side and reduced forward scatter values compared with those of living cells. Anti-HIV activities were determined in at least three independent experiments, performed in triplicate. To evaluate the anti-HIV activity in cell-cell transfer, overnight cocultures between isolated primary CD4+ T cells (2×10^5) and uninfected or infected$MOLT_{NL4-3}$ cells (2×10^5) were performed in the presence of serial dilutions of the corresponding test compounds. Virus transfer was measured as described above. The 50% effective concentration (EC_{50}) and the 50% cytotoxic concentration (CC_{50}) were calculated for cell free-virus infections and cell-cell CAp24 antigen transfer. Bafilomycin A1 (BFLA1) and concanamycin A (CON A) were purchased from Sigma.

Infection with Viruses Released from Antigen-loaded Cells. Cocultures between freshly isolated primary CD4+ T cells and uninfected or infected $MOLT_{NL4-3}$ cells were performed as described above. After 6 h of coculture, to minimize the possibility of CD4+ T cell infection, target cells were sorted (>99% purity) as indicated above, and

recovered target cells were cultured (5×10^5 cells/condition) in the presence or the absence of 80 nM IgGb12 to prevent productive infection. After 12 h of culture, the presence of CAp24-antigen was evaluated both in the supernatant and in the purified cells by intracellular CAp24 antigen staining as indicated above. Total viral DNA was also quantified by PCR as indicated below using infected CD4+ T cells as a positive control. For each condition, 20 µl of supernatant was used to infect 3×10^4 MT4 cells for 5 days. Infection of MT4 T cells was evaluated by quantification of supernatant CAp24-antigen content.

Quantitative Real-time PCR for Total HIV-1 DNA Detection. Total DNA was quantified as described before [94, 95]. Briefly, purified CD4+ T lymphocytes were centrifuged, supernatant was removed, and pellets were frozen. Total cellular DNA was extracted using QIAamp DNA extraction kit (QIAamp DNA Blood mini kit; Qiagen) as recommended by the manufacturer. Quantitative amplification of LTR for viral entry detection was performed using the following primers and probe (forward, 5'-GACGCAGGACTCGGCTTG-3'; reverse, 5' ACTGACGCTCTCGCACCC-3' and probe 5'-TTTGGCGTACTCACCAGTCGCCG-3' labeled with the fluorophore FAM and the quencher TAMRA). To normalize HIV copy values/cell, amplification of cellular RNaseP gene was performed using TaqMan® RNaseP Control Reagents Kit (Applied Biosystems). DNA extracted from 8E5/LAV cells (harboring one copy of integrated HIV-1/cell) was used to build a standard curve. The PCR was performed in a total volume of 50 µl using 1 × TaqMan® Universal PCR Master Mix (Applied Biosystems, Roche), 0.9 µM concentration of the primers, 0.25 µM probe, and 5 µl of the DNA sample. Reactions were analysed with the ABI PRISM 7000 instrument using SDS 1.1 software (Applied Biosystems). For each condition, the amount of the total viral DNA/cell was normalized to untreated sample with IgGb12, and results are expressed as the relative percent increase.

Virus-Cell Fusion Assay. The quantification of the virus-cell membrane fusion was quantified as described before [96]. Briefly, 1×10^5 HEK293-T cells were cotransfected with 0.4 µg of both the NL4-3 HIV provirus plasmid (pNL4-3 from the AIDS Reagents Program) and a plasmid carrying the *Vpr* gene fused with β-lactamase (*Vpr*-BlaM; pMM310 from the AIDS Reagents Program). After 48 h, transfected HEK293-T cells were cocultured overnight with primary CD4+ T lymphocytes as described above. Cells were then recovered and loaded with the CCF2-AM loading kit (Invitrogen) following

the protocol provided by the manufacturer. Cells were incubated for 1 h at room temperature, then washed and immediately fixed. The change in emission of the cleaved CCF2 generated by the *Vpr*- BlaM chimera was measured by flow cytometry.

Evaluation of Dynasore Activity. Primary CD4+ T lymphocytes were pretreated with or without different concentrations of dynasore starting at 160 μM, for 30 min at 37 °C. Then, pretreated CD4+ T lymphocytes were cultured in the presence or the absence of phorbol 12-myristate 13 acetate (PMA; Sigma) at 1 μg/ml for 30 min at 37 °C. Cells were fixed with 1% formaldehyde and after washes with PBS, stained for CD4 expression with anti-CD4 mAb conjugated with the fluorochrome FITC (BD Bioscience). Analysis of cells was performed by flow cytometry.

Immunofluorescence, Confocal Microscopy, and Quantification of Colocalization. For immunofluorescence staining, cocultures of primary CD4+ T cells with uninfected or infected MOLTNL4-3 cells were performed as described above. Samples were trypsinized to remove potentially bound viruses into the cell surface and after subsequent washes with PBS cells were fixed, permeabilized (Fix & Perm), and incubated for 1 h at room temperature with the anti CAp24 mAb KC57-FITC (Coulter) and the CD4-PE (BD Bioscience) or with the goat anti-human-dynamin antibody (clone N-19, Santa Cruz Biotechnology). For dynamin staining, cells were then washed and incubated for 1 h at room temperature with the donkey antigoat Alexa Fluor 647-conjugated secondary antibody (Molecular Probes, Invitrogen). Cells were adhered onto glass slides using cytospins (Thermo Scientific) and mounted with Prolong Gold antifade reagent (Invitrogen). Images were acquired on a Leica TCS SP5 AOBS confocal microscopy (Leica Microsystems CMS GmbH, Mannheim, Germany). Z-sections were acquired at 0.5-μm steps using an Argon 488/458 and HeNe 633 lasers and a plan Apochromat 63 × 1.4 oil objective, supplied with the imaging software LAS AF (Leica Microsystems). Determination of the colocalization coefficient between CAp24 protein and the CD4 receptor or dynamin protein was performed using single Z-stacks and evaluated with LAS AF software.

Statistical Analysis. Student's *t* test was used to determine statistical significance (**, $p < 0.005$ or *, $p < 0.05$) between values.

1.3.- Results

1.3.1.- HIV Transmission during Cell-to-cell Cocultures

Overnight cocultures of HIV-1 NL4-3 persistently infected MOLT-4/ CCR5 cells ($MOLT_{NL4-3}$) with CMFDA-loaded nonstimulated primary CD4+ T lymphocytes were evaluated by flow cytometry. After overnight coculture, intracellular staining of capsid p24 (CAp24) HIV antigen was detected in 23% of target cells (**Figure 5**). The transfer of viral antigens to uninfected cells was clearly blocked by the neutralizing anti-gp120 mAb IgGb12 (95% of inhibition compared with the untreated condition), but was not inhibited by the RT inhibitor AZT or the dynamin-dependent endosome-scission inhibitor dynasore (21 and 22% of p24+ cells, respectively) despite using high drug concentrations (2000 fold higher than the EC_{50} of AZT under cell-free infection conditions, **Table 1**). Macrolide antibiotics such as BFLA1 and CON A that prevent endosome and lysosome acidification did not have any effect on virus uptake (**Figure 5B**).

Interestingly, in the presence of the coreceptor antagonist AMD3100 the uptake of HIV particles by the target cells increased roughly 3-fold compared with untreated condition (65% of target cells were positive for CAp24 antigen staining) even when cells were cocultured with ~700-fold higher EC_{50} (**Table 1**). Taken together, these results confirmed that cellular contacts between infected lymphoid cells and primary CD4+ T lymphocytes triggered CD4-dependent transmission of high amounts of HIV-1 particles from infected to uninfected cells.

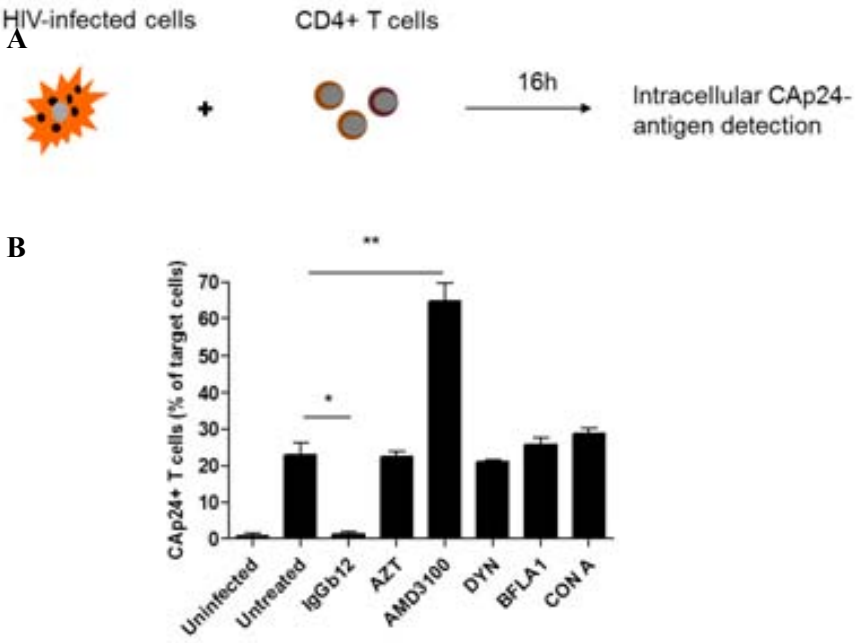


Figure 5 . CD4-dependent tr ansfer of H IV antigen a fter c ell-to-cell co ntacts. A. Experimental procedure was o vernight cocultures o f $MOLT_{NL4-3}$ cells with p rimary C D4+ T l ymphocytes. **B.** Cocultures were performed in the presence of 80 nM anti-gp120 mAb IgGb12; 4 µM RT inhibitor AZT; 12.5 µM CXCR4 antagonist AMD3100; 80 µM dynamin inhibitor dynasore (*DYN*); 100 nM BFLA1, and 20 nM CON A. Results are represented as the percentage of intracellular CAp24+ target cells, using the coculture between CD4+ T cells and uninfected MOLT cells as a negative control. Results are the mean ± S.D. (*error bars*) of three independent experiments (**, $p < 0.005$; *, $p < 0.05$).

Table 1

Potent postattachment inhibitors of HIV replication do not block cell-to-cell transfer of virus

	Anti-HIV-1 activity		Cell-to-cell HIV-1 Transfer	
Compound	**EC_{50} [a]**	**CC_{50} [b]**	**EC_{50} [c]**	**CC_{50} [b]**
	µM	µM	µM	µM
AMD3100	0,018 ± 0,0023	> 0,125	No effect at 62 µM	> 62
AZT	0,0021 ± 0,0003	> 0,4	No effect at 20 µM	> 20
IgGb12	0,0003 ± 0,0001	> 0,04	0,0006 ± 0,0001	> 0,4
Dynasore	No effect at 40 µM	40	No effect at 80 µM	250

[a] EC_{50}: Effective concentration needed to inhibit 50% replication of the wild-type HIV-1NL4–3 strain in peripheral blood mononuclear cells.
[b] CC_{50}: Cytotoxic concentration n eeded t o induce 5 0% d eath o f n oninfected cel ls, evaluated b y morphology changes using flow cytometry 7 days after infection.
[c] EC^{50}: Effective c oncentration n eeded to b lock 5 0% o f HIV -1NL4-3-antigen transfer in CD4+ T cells determined b y in tracellular CAp24 a ntigen sta ining a fter overnight cocultures between HIV -infected $MOLT_{NL4-3}$ cells and primary CD4+ T cells.
[d] CC^{50} evaluated after overnight cocultures.

To e valuate virus-cell f usion, H IV-1 NL4-3 tra nsfected *Vpr*-BlaM+ HE K293-T c ells were c ocultured with target CD4+ T c ells and fusion wa s measured b y de tection of cleaved C CF2. As expected, mAb IgGb12 completely block ed virus -dependent fusion similar to the observed i nhibition of virus c apture (**Figure 6**). C onversely, AMD3100 blocked virus-cell fusion (**Figure 6**) although it did not block but significantly increased

virus transfer (**Figure 5**). AZT or d ynasore did not prevent cleavage of C CF2, suggesting that vir us antigen was passively transferred to CD4+ T cells in the absence of virus cell fusion as noted in the AMD3100-treated cells.

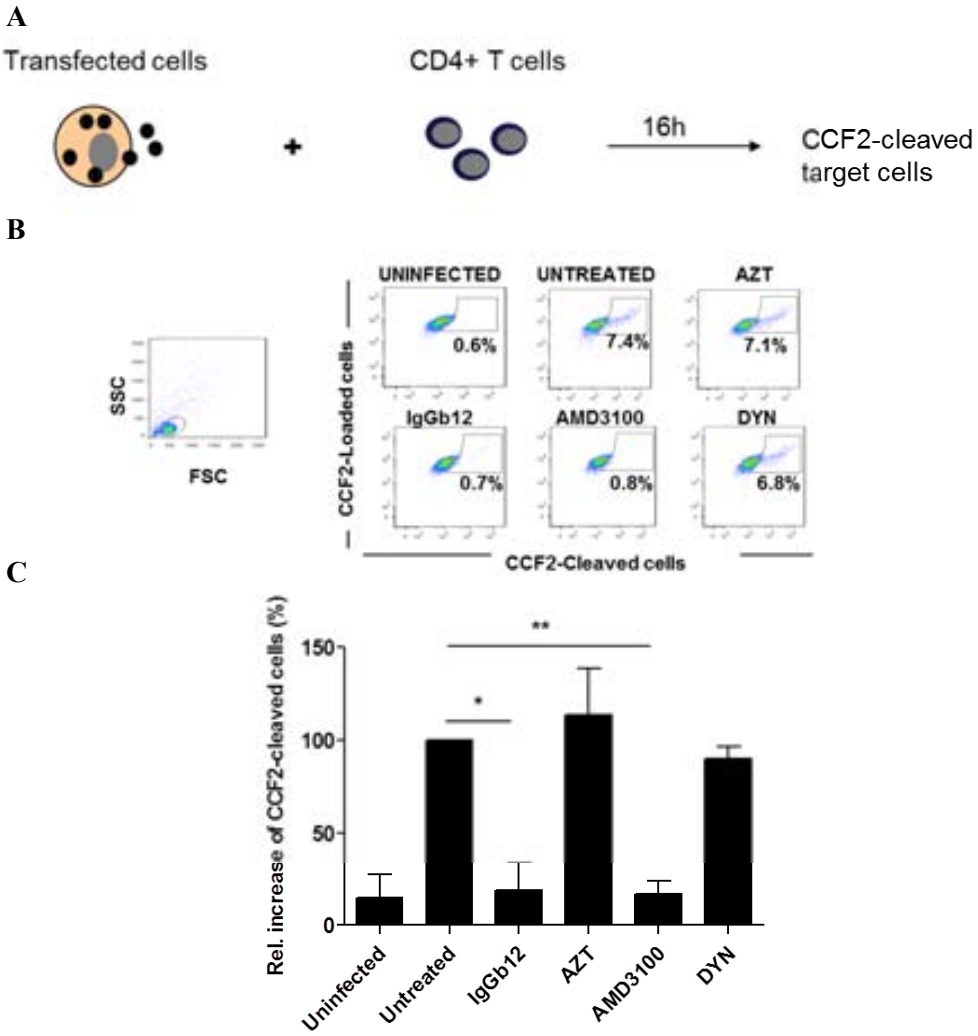


Figure 6. IgGb12 and AMD3100 but not dynasore blocked virus-cell fusion after cell to cell transfer of v irus. A. Experimental p rocedure: measurement of v iral fusion i n c ocultures of HEK2 93-T cel ls transfected w ith pNL4-3 a nd *Vpr*-BlaM plasmids and p rimary C D4+ T c ells. **B.** Dot plots o f CCF2-loaded cells (F ITC-labeled) *versus* CCF2-cleaved cells (*Pacific b lue*-labeled). A representative experiment is sh own. **C.** Relative in crease o f CCF 2-cleaved targ et cells compared w ith u ntreated condition. Data are the mean ± S.D. (*error bars*) of three independent experiments (**, $p < 0.005$; *, $p < 0.05$) (*DYN*, dynasore).

1.3.2.- Productive Infection Did Not Occur from within Intracellular Compartments

We h ypothesize that int ernalized virus a fter cell-to-cell tra nsfer could not fuse fr om within intracellular compartments. To evaluate the fate of internalized HIV-1 particles

captured by CD4+ T cells after cell-to-cell transfer, CMFDA-loaded target CD4+ T cells were purified from infected $MOLT_{NL4-3}$ lymphoid cells by fluorescence-activated cell sorting (>99% purity). Following separation purified CD4+ T cells were trypsinized to eliminate virus bound to the cell surface. Trypsin treatment dramatically reduced the expression of CD4 in purified T cells; however, CAp24 antigen staining was not significantly reduced (data not shown), suggesting that captured virus resided in intracellular compartments. Immediately after washings, for each initial coculture condition, target cells were subdivided in three and left in culture during 5 days in drug-free medium or in the presence of the mAb IgGb12 or IgGb12+AZT (**Figure 7A**). Drug concentrations used clearly ensure complete inhibition of infection (300-fold and 2000-fold higher EC_{50} for IgGb12 and AZT, respectively, **Table 1**). Dynasore was not included as it was cytotoxic in long term cultures (data not shown). Virus production is low to undetectable in nonstimulated cells [36, 97, 98]; thus, PHA/IL-2 was added to the medium to promote virus replication. After 5 days in culture, CAp24 antigen in cell supernatant (**Figure 7B**) and total viral DNA detection by quantitative PCR (**Figure 7C**) were evaluated as a measure of virus replication and indicated that antigen-loaded cell cultures became productively infected after PHA/IL-2 activation in the absence of inhibitors in the culture medium (**Figure 7, grey bars**). Virus production was in concordance to the amount of virus transferred during the coculture phase (**Figure 5**). Thus, in the absence of antigen transfer (IgGb12-treated coculture), no virus production was found. Conversely, the high uptake of CAp24-antigen in the AMD3100-treated cocultures coincided with an increase in virus production in purified cells. The RT inhibitor AZT did not prevent virus transfer or fusion and partially blocked supernatant CAp24 antigen production or total DNA detection as a consequence of being present only during the coculture phase. However, when IgGb12 was present during the purified cell culture phase (**Figure 7, black and white bars**), virus replication was significantly blocked irrespective of the condition used during the coculture phase (**Figure 7, *x* axis, angled labels**). Taken together, these results indicate that the conditioned medium with IgGb12 prevented internalized virus particles from initiating a productive infection. Virus needed to reach the extracellular environment to initiate a productive infection, an event that could only occur when the attachment inhibitor, mAb IgGb12, was not present.

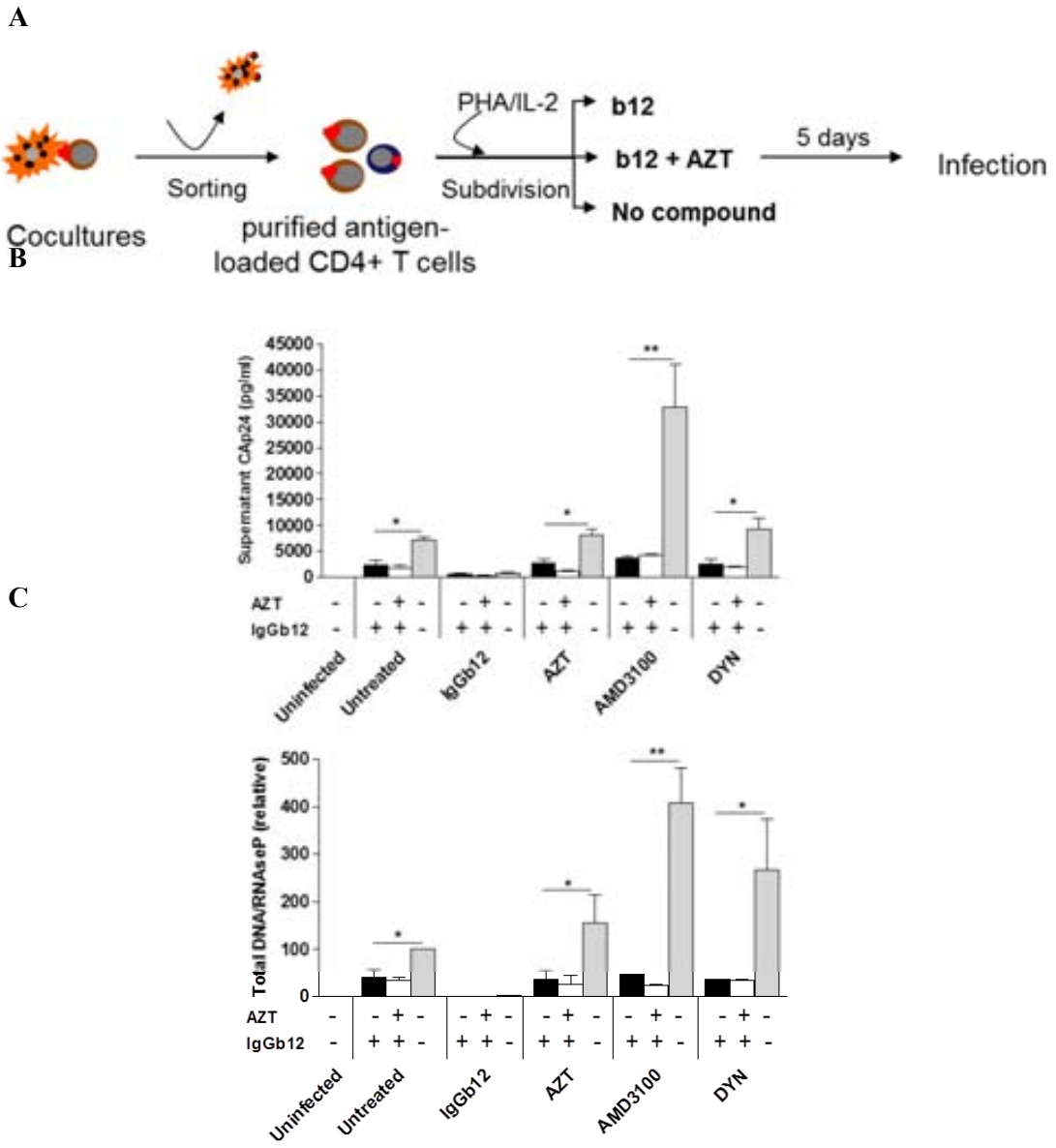


Figure 7. Infection of CD4+ T cells by HIV particles captured into trypsin-resistant compartments was inhibited by mAb IgGb12. A. Experimental procedure: isolation and culture of CAp24-loaded CD4+ T cells. After 5 days of culture, HIV infection was assessed by supernatant CAp24 antigen production, expressed in pg/ml (**B**) and quantification of total viral DNA as the copy number of total DNA/RNaseP, expressed relative to the untreated condition (cells untreated during the coculture and culture phase) (**C**). Results represent the mean ± S.D. (*error bars*) of three independent experiments (**, $p < 0.005$; *, $p < 0.05$) (*DYN*, dynasore).

1.3.3.- Infection by HIV Particles Released from Antigen-loaded Cells

Our results suggest that the inability of virions to infect cells from within endosomal compartments could promote the recycling of HIV particles to the cell surface that could later infect bystander cells. To further explore this hypothesis, antigen loaded primary CD4+ T lymphocytes were sorted after short term cocultures (6 h) with infected

$MOLT_{NL4-3}$ cells (**Figure 8A**). IgGb12-treated coculture, in which CAp24-antigen transfer was completely blocked, was used to control the effect of contaminant MOLTNL4-3 cells (<0.1%). Once purified, antigen loaded CD4+ T cells were left in culture for 12 h in the presence or the absence of IgGb12 to restrict reinfection events while allowing release of virions in the supernatant. The CAp24-antigen found in the supernatant was concordant with the level of intracellular CAp24-antigen in loaded target cells (**Figure 8B**). Total DNA in purified target cells was measured to ensure that antigen-loaded cells did not become infected during the culture (**Figure 8C**). Compared with infected control cells, target cells remained negative, suggesting that particles found in the supernatant did not come from new infection events but released from endocytic compartments. Supernatants were collected after 12 h and used to infect lymphoid MT4 T cells (**Figure 8D**). The supernatants from untreated and AMD3100-treated cultures were able to establish a productive infection in MT4 cells. Conversely, the supernatant of the IgGb12 condition could not infect target cells, indicating that infection was not generated from contaminant $MOLT_{NL4-3}$ cells. These results indicate that antigen-loaded cells did not become infected but were able to infect bystander CD4+ cells after recycling of HIV to the cell surface and release to the cell supernatant.

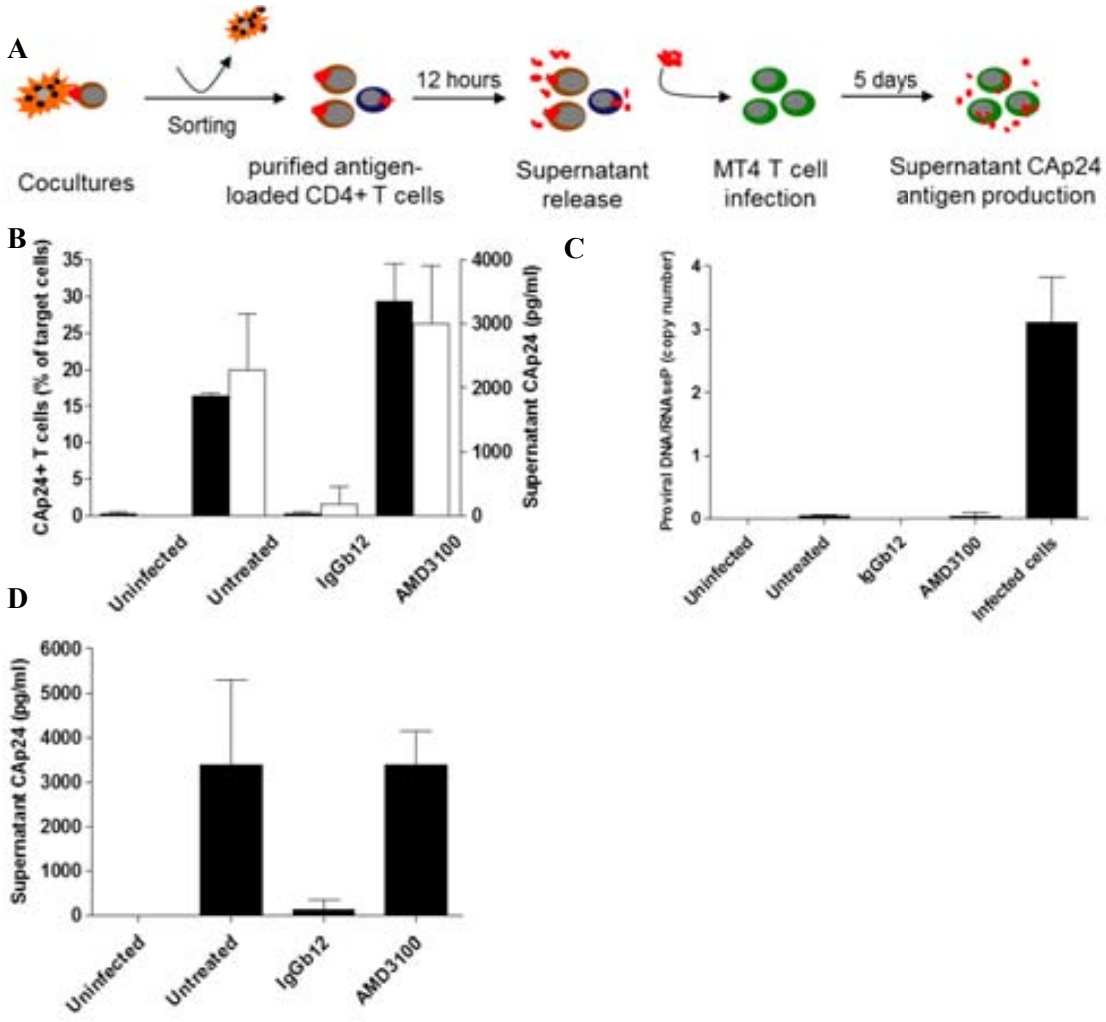


Figure 8. Trans-infection by released HIV viruses from antigen-loaded cells. A. In this experimental procedure, supernatants from cocultures were collected and used to infect MT4 T cells. **B.** After 12 h of culture, p24-antigen content was evaluated in the supernatant (*white bars*) and in the purified cells (*Black bars*) by CAp24 ELISA and intracellular CAp24 antigen staining, respectively. **C.** Total viral DNA was also quantified in purified cells by PCR using infected CD4+ T cells as a positive control. Results represent the total viral DNA copy number relative to the cellular control gene RNaseP. **D.** Infection of MT4 T cells by collected supernatants was evaluated at day 5 by supernatant CAp24-antigen content. Data are the mean ± S.D. (*error bars*) of three independent experiments.

1.3.4.- Dynasore Did Not Block Uptake or Infection of CD4+ T Cells

Dynasore (80 µM), a dynamin dependent endosomal scission inhibitor, has been shown to block the infection of HeLa cells, suggesting that endosomal uptake was a prerequisite for fusion and infection (20). We and others have shown that cell-to-cell transfer of HIV may occur through an endocytic process in which virus antigen is colocalized with clathrin and dynamin [19, 32, 35, 36, 88]. However, dynasore did not prevent the CD4-dependent uptake of HIV antigen into target cells (**Figure 5**), did not prevent virus replication in antigen-loaded, activated cells after cell-to-cell transfer of

virus (**Figure 7**), and was devoid of antiviral activity in peripheral blood mononuclear cells at subtoxic concentrations (**Table 1**). Conversely, dynasore blocked the PMA-induced down-regulation of the CD4 receptor in primary CD4+ lymphocytes (**Figure 9**), a process that involves a clathrin dependent endocytic pathway [99].

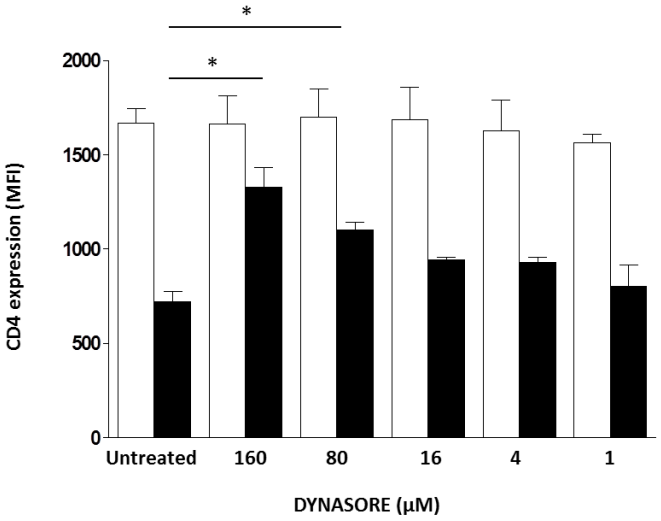


Figure 9. Dynasore prevents PMA-induced down-regulation of CD4 receptor. Primary CD4+ T lymphocytes were pretreated for 30 min with or without 160, 80, 16, 4, and 1 µM of dynasore and then cultured in the absence (*white bars*) or the presence of PMA at 1 µg/ml (*black bars*) for an additional 30 min. Then, cells were fixed with 2% of formaldehyde, and surface CD4 expression (mean fluorescence intensity, *MFI*) was evaluated with an anti-CD4 mAb. Cells were analysed by flow cytometry and identified by morphology. Dynasore inhibited PMA-induced CD4 down-regulation in a dose-dependent manner. Data are the mean ± S.D. (*error bars*) of three independent experiments. *, $p < 0.05$.

To analyse the effect of dynasore in dynamin function during HIV uptake we performed a colocalization analysis between HIV Gag antigen (CAp24) and dynamin in untreated or dynasore-treated cocultures (**Figure 10 A**). Colocalization coefficients of 0.73 and 0.75 between CAp24 antigen and dynamin protein were calculated in both untreated and dynasore-treated cocultures respectively, indicating that early compartmentalization of HIV particles was associated with the dynamin endocytic machinery, but could not be blocked by an agent targeting the scission of early formed endosomes. Colocalization between HIV Gag antigen (CAp24) and CD4 receptor in untreated or dynasore-treated cocultures between primary CD4+ T cells and infected $MOLT_{NL4-3}$ cells showed similar colocalization coefficients (0.78 and 0.84 for untreated and dynasore-treated conditions, respectively) (**Figure 10B**).

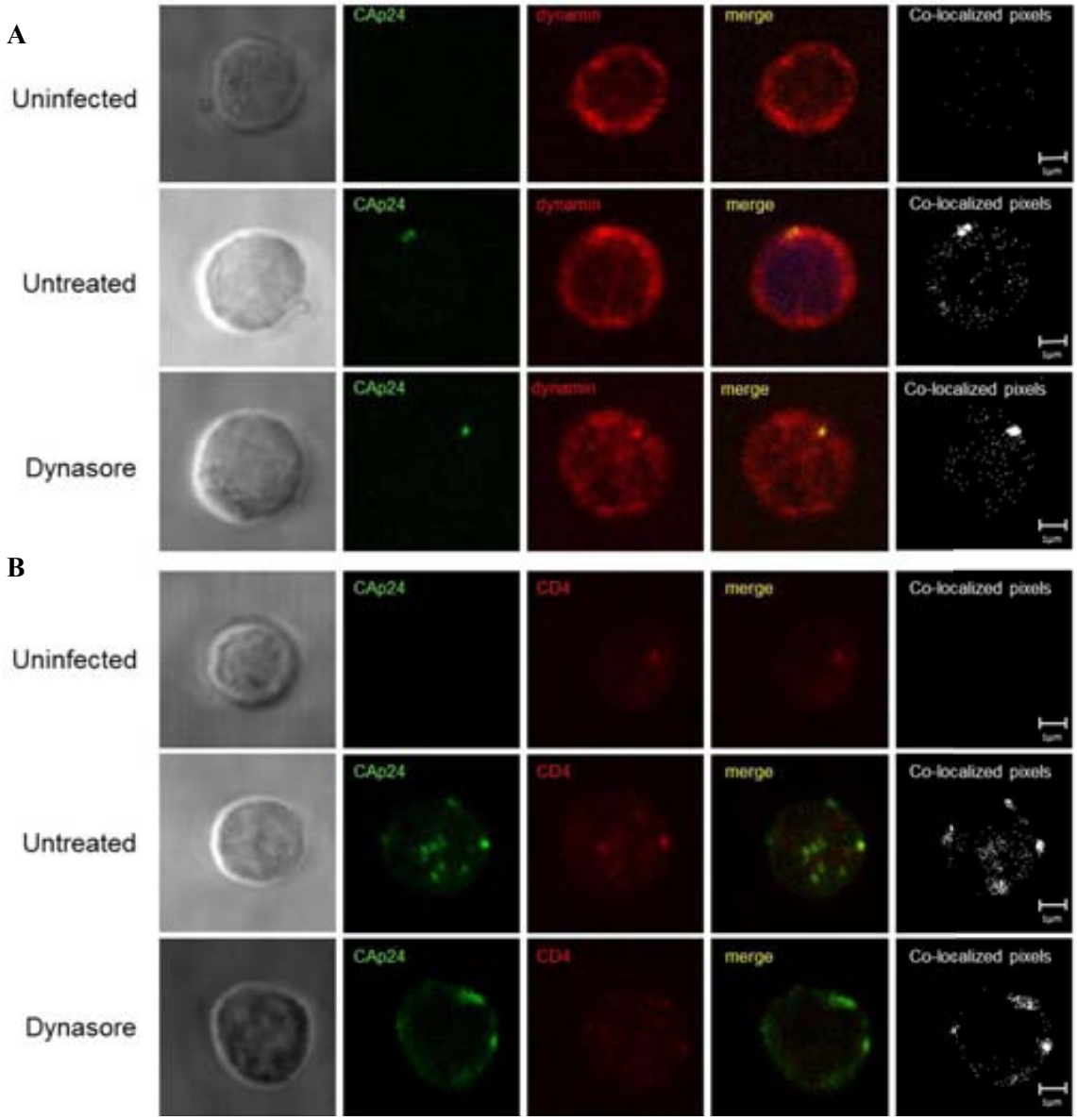


Figure 10. Uptake of HIV particles into intracellular CD4+ compartments in primary T lymphocytes was not blocked by dynasore. Primary CD4+ T lymphocytes were cocultured overnight with HIV-infected $MOLT_{NL4-3}$ cells in the presence or the absence of dynasore (80 µM). Recovered cells were trypsinized to remove membrane-bound viruses and immunostained with antibodies against HIV CAp24 antigen, CD4 receptor, and dynamin. Sections of single target CD4+ T cells were viewed and analysed by confocal microscopy. Colocalization between (**A**) HIVp24 antigen (*green*) and dynamin protein (*red*) or between (**B**) HIVp24 antigen (*green*) and CD4 receptor (*red*) was performed for uninfected (*upper panels*), untreated (*middle panels*), and dynasore-treated (*lower panels*) cocultures. The images show the phase-contrast (*left column*), the single stainings, the overlay (*yellow*), and the colocalized pixels (*white*). A CD4+ T lymphocyte representative of each coculture is shown from at least two independent experiments.

1.4.- Discussion

Complete fusion of HIV particles within endosomal membranes has been used to indicate that internalization of HIV particles through an endocytic pathway was required for infection [54, 91]. Here, we show, using primary CD4 T lymphocytes that cell-to-cell contacts between HIV infected and uninfected cells induced the endocytic uptake of viral particles into trypsin resistant, dynamin-enriched compartments. Only the inhibition of gp120-CD4 interaction (virus attachment to CD4) could block the transfer of HIV particles. Conversely, the addition of the coreceptor inhibitor AMD3100 induced the accumulation of virus particles leading to massive endocytosis into cells in which the virus-cell fusion process was completely arrested [35, 36, 46]. Activation of purified antigen-loaded cells initiated a productive infection but only when cells were cultured in the absence of mAb IgGb12, an inhibitor of virus attachment to CD4. IgGb12 should be unable to penetrate the cell surface. However, we cannot completely exclude the possibility of an antibody such as IgGb12 to enter already formed intracellular compartments containing HIV particles.

These results suggest that endocytosed viral particles could not initiate a productive infection from within endosomes in primary CD4+ T cells (*i.e.* by virus fusion to the endosomal membrane). We hypothesize that endocytosed viruses could only induced infection in *trans* (trans-infection) because they were required to resurface and reach the extracellular environment and engage CD4 leading to virus-cell fusion and replication, a condition that could only be achieved in the absence of an attachment inhibitor in the cell supernatant. We have shown that antigen-loaded cells may release virus particles [51], and cocultures of antigen-loaded T cell with U87-CD4 target cells may lead to infection of the U87-CD4 cells [36], indicating the possibility of trans-infection. Here, we demonstrate that supernatant from purified antigen-loaded, but viral DNA-negative T cells, released virus to the supernatant that later infected MT4 cells, strongly suggesting that antigen-loaded cells trans-infect bystander CD4+ T cells.

Recent data indicate that prior to membrane fusion, virions may need to undergo maturation after cell-to-cell transfer of HIV-1 [46], a process that might be impaired or further delayed in nonstimulated primary CD4+ T cells, and thus, productive infection was only possible after virus recycling to the cell surface. Moreover, virion maturation may allow the virus to transfer from cell-to-cell in a conformation immunologically

distinct that might escape the detection by neutralizing antibodies. However, these findings are in contrast to data showing that anti-gp41 antibodies 4E10 and 2F5 did not block the transfer of HIV particles from infected to target cells but blocked productive infection of target cells [52], suggesting that HIV infection between T cells is transmitted by a neutralization-sensitive mechanism [52, 53]. Our results reinforce the idea that endocytosed virus after cell-to-cell contacts may represent an itinerant virus reservoir able to induce the trans-infection of bystander T cells, but not leading to effective virus fusion or replication from within internal endosomal compartments. The contribution of this mechanism in the pathogenesis of HIV *in vivo* still needs to be completely clarified but should be taken into account when developing new antiviral strategies [100].

Using confocal microscopy, we found clathrin and dynamin proteins colocalized with HIV particles [35] which in turn were colocalized with CD4 (**Figure 10**). However, dynasore, a dynamin-dependent endosomal scission inhibitor previously shown to block virus replication in HeLa cells [54, 91], did not prevent virus capture, virus cell fusion, and virus replication after cell-to-cell transfer to primary CD4_ T cells. In concordance, previous observations indicated that VSV-G pseudotyped HIV infection could not be inhibited after dynasore treatment, suggesting that VSV and HIV envelopes mediate distinct modes of virus entry [55]. Moreover, it has been demonstrated that dynasore inhibits clathrin-mediated endocytosis at two different steps. The ultrastructural analysis of the effect of dynasore on clathrin-coated structures shows the appearance of “U” and “O” shape-coated pits associated with the plasma membrane [101]. Consequently, internalization of CAp24 antigen into the “initial” coated pits in the presence of dynasore cannot be ruled out. Altogether, it appears that internalization of particles initially required the endocytic machinery, and dynasore might not be able to inhibit the initial formation of these endocytic compartments. Blocking HIV endocytosis (*e.g.* with dynasore) without preventing virus replication would be the ultimate proof of endocytosis not being necessary for infection. This could not be achieved with dynasore at nontoxic concentrations, and therefore, the hypothesis remains unresolved. However, we clearly show that internalized virus required to resurface to initiate a productive infection, suggesting that endocytosis may not be a route of productive infection.

The development of new small molecule inhibitors of clathrin-coated pit assembly (Pitstop) allowed better characterization of clathrin functions within the endocytic network [102]. Pitstop induced inhibition of clathrin terminal domain interferes with receptor-mediated endocytosis and synaptic vesicle recycling and has been shown to increase the lifetime of clathrin-coated components, including dynamin. These agents were also shown to block HIV entry in HeLa cells, but it remains to be resolved whether inhibition of virus replication was due to preventing virus-endosome fusion or the recycling of HIV particles. Importantly, the antiviral activity of endosome function should be evaluated in primary T cells that better model the interactions between virus and cell functions.

Nef-induced down-regulation of CD4 results in internalization and degradation of surface CD4 in lysosomes [103]. Prevention of endosome and lysosome acidification by macrolide antibiotics such as BFLA1 and CON A inhibits degradation of CD4and consequently promotes accumulation of CD4 in endosomes and lysosomes [104]. Moreover, different types of endosome acidification inhibitors increase infectivity of HIV particles presumably by preventing them from degradation in late endosomes and lysosomes [105]. Colocalization of HIV particles with CD4 in dynamin-containing endosomes could indicate that CD4 protects virus particles from degradation and helps recycle back HIV to the cell surface. However, in our hands, acidification inhibitors such as BFLA1 and CON A did not prevent or augment virus transfer from infected to uninfected cells. Therefore, our results did not shed light on the protective role of CD4 in endocytic virus degradation.

In conclusion, after cell-to-cell transfer of HIV-1 into target primary CD4+ T cells we observed that cells were only infected if left in culture in the absence of an attachment inhibitor to CD4 (mAb IgGb12), suggesting that virus needed to resurface to begin a productive infection. Moreover, dynasore, an inhibitor of dynamin-dependent endocytosis, did not block virus replication. Endocytosis may not be the primary mechanism of infection by HIV-1 after cell-to-cell contact, but a reservoir able to induce trans-infection of bystander CD4+ T cells.

1.5.- Acknowledgments

We thank the National Institutes of Health (AIDS Research and Reference Reagent Program) and the EU Programme EVA Centralised Facility for AIDS Reagents, National Institute for Biological Standards and Control, United Kingdom, for reagents. We thank Marco A. Fernández (Institut Germans Trias i Pujol, Badalona, Spain) for technical assistance.

Chapter 2.- Antiretroviral Agents Effectively Block HIV Replication after Cell-to-Cell Transfer

Marc Permanyer, Ester Ballana, Alba Ruiz, Roger Badia, Eva Riveira-Munoz, Encarna Gonzalo, Bonaventura Clotet, and José A. Esté

IrsiCaixa, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Spain

J Virol. 2012 Aug;86(16):8773-80.

Cell-to-cell transmission of HIV has been proposed as a mechanism contributing to virus escape to the action of antiretrovirals and a mode of HIV persistence during antiretroviral therapy. Here, cocultures of infected HIV-1 cells with primary CD4+ T cells or lymphoid cells were used to evaluate virus transmission and the effect of known antiretrovirals. Transfer of HIV antigen from infected to uninfected cells was resistant to the reverse transcriptase inhibitors (RTIs) zidovudine (AZT) and tenofovir, but was blocked by the attachment inhibitor IgGb12. However, quantitative measurement of viral DNA production demonstrated that all anti-HIV agents blocked virus replication with similar potency to cell-free virus infections. Cell-free and cell-associated infections were equally sensitive to inhibition of viral replication when HIV-1 long terminal repeat (LTR)-driven green fluorescent protein (GFP) expression in target cells was measured. However, detection of GFP by flow cytometry may incorrectly estimate the efficacy of antiretrovirals in cell-associated virus transmission, due to replication-independent Tat-mediated LTR transactivation as a consequence of cell-to-cell events that did not occur in short-term (48-h) cell-free virus infections. In conclusion, common markers of virus replication may not accurately correlate and measure infectivity or drug efficacy in cell-to-cell virus transmission. When accurately quantified, active drugs blocked proviral DNA and virus replication in cell-to-cell transmission, recapitulating the efficacy of antiretrovirals in cell-free virus infections and *in vivo*.

2.1.- Introduction

Antiretroviral therapy (ART) and human immunodeficiency virus research have achieved unprecedented series of breakthroughs that have translated into the largely successful management of what is now considered a chronic treatable infection [106, 107]. In long-term-treated patients, the withdrawal of ART leads to a rebound of the plasma viral load, indicating that current treatment is unable to eradicate the virus from an infected patient despite apparently suppressed viral replication. Multiple factors may affect HIV persistence in the presence of antiretroviral treatment. Persistent viremia may arise from long-lived productively infected cells that were infected prior to therapy initiation or from the intermittent reactivation of latently infected cells. Alternatively, residual viral replication during ART or in sanctuary sites into which antiretrovirals poorly penetrate would explain HIV persistence [108]. The degree to which HIV can

effectively replicate during therapy is a highly contentious issue [109], and it is actively being studied in patients and cell culture models.

Recently, Sigal et al. [5] suggested that cell-to-cell transmission of HIV represents a mechanism contributing to virus escape from the action of antiretrovirals and a mode of HIV persistence during antiretroviral therapy. A mathematical model was used to demonstrate that the drug concentration required to prevent a single transmitted virion from infecting a target cell is much lower than that needed to stop multiple transmitted virus particles from infecting the same cell. Sigal et al. defined a transmission index (*Tx*) as the ratio between the number of infected cells in the presence of drug (*Id*) and the fraction of cells infected in the absence of drug (*I*), adding further complexity to the model by associating *Tx* with the multiplicity of infection (MOI; abbreviated as "*m*" in the equation presented below), defined as the product of virus particle number and the infectivity per virus particle. Then, cell-to-cell spread was used as the experimental model to recapitulate the effect of multiple virus transmission.

We and others have demonstrated that coculture of HIV-1-infected cells with CD4+ T cells leads to detection of large amounts of enveloped virions in clathrin-coated endosomes [19, 32, 35, 36, 54, 82, 110] that persist for 48 h [36] or as long as the target cells are cultured with infected cells. Cell-to-cell HIV transfer is blocked by agents that prevent virus attachment [51], but is resistant to agents targeting HIV coreceptors, gp41-dependent fusion inhibitors or neutralizing antibodies, and, importantly, agents targeting the reverse transcriptase (RT) [52, 82, 88]. Endocytic transfer of HIV may lead to virus replication, but incoming viral antigen in target cells may incorrectly be interpreted as a marker of virus replication. Therefore, the aim of the present work was to evaluate the efficacy of known antiretrovirals in cell-associated virus transmission compared to cell-free virus infection. We show that anti-HIV drugs are able to block viral DNA production and the inherent virus replication in cell-to-cell viral transmission, recapitulating the efficacy of antiretrovirals in cell-free virus infection.

2.2.- Materials and Methods

Cells. Peripheral blood mononuclear cells (PBMCs) from healthy donors were purified by Ficoll-Hypaque sedimentation. CD4+ T lymphocytes were immediately purified (>95%) from PBMCs by negative selection using the CD4+ T cell enrichment kit (Stem

Cell Technologies, Vancouver, Canada) and grown in RPMI 1640–L-glutamine medium (Gibco, Madrid, Spain). Chronically HIV-1-infected MOLT cells were generated after infection of the MOLT-4/CCR5 lymphoid cell line (AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, MD) with an NL4-3 X4 HIV-1 strain (HIV-1_{NL4-3}) constructed in an HIV_{HXB2} backbone [111]. After the infection peak, the persistently infected culture was grown and characterized for Env expression and virus production [112]. Uninfected MOLT-4/CCR5 cells were used as negative controls in all experiments. MOLT-4, the lymphoid T cell line MT-4 (obtained from the Medical Research Council, Centre for AIDS Reagents, London, United Kingdom), and the green fluorescent protein (GFP) expression CEM GFP cell line (AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, MD) were cultured in RPMI (Gibco, Madrid, Spain). Media were supplemented with 10% heat inactivated fetal calf serum (FCS; Life Technologies, Madrid, Spain), 100 U/ml penicillin, and 100 µg/ml streptomycin.

Cocultures of infected and uninfected cells. Primary CD4+ T cells were cocultured with uninfected or HIV-1 persistently infected MOLT cells as previously described [36, 51, 88]. Briefly, target cells (purified primary CD4+ T cells or MT-4 cells) were first labeled with the cell tracer DDAO-SE (Molecular Proves, Life Technologies) at a final concentration of 0.5 µg/ml for 30 min. Cells were extensively washed before being mixed with effector MOLT cells. Both effector and target cells (2×10^5 cells each in a 1:1 ratio) were cocultured in a 96-well culture plate in a final volume of 0.2 ml in the absence or the presence of the following HIV-1 inhibitors: the reverse transcriptase inhibitors (RTIs) zidovudine (AZT; 4 µM) (Sigma- Aldrich) and tenofovir disoproxil fumarate (TDF; 4 µM) (AIDS Research and Reference Reagent Program) and 10 µg/ml of the neutralizing anti-gp120 monoclonal antibody (MAb) IgGb12 (Polymun Scientific). After overnight coculture, primary CD4+ T cells were purified (>99% purity) from MOLT cells by fluorescence-activated cell sorting (FACS) (FACSAria II; BD Biosciences). Recovered CD4+ T cells were maintained in the presence of the same compound used during the initial coculture. After 5 days, infection in target cells was assessed by enzyme-linked immunosorbent assay (ELISA) for HIV capsid (CA) p24 antigen detection in culture supernatants (Genscreen HIV-1 Ag EIA; Bio-Rad Laboratories). Cocultures with target MT-4 T cells were performed during 2 h, 8 h, 24 h, and 48 h at 37°C.

Evaluation of HIV transfer. The capture of HIV-1 p24 antigen (p24) by primary CD4+ T cells and MT-4 cells was evaluated by flow cytometry as shown before [32, 35]. Prior to staining, cells were trypsinized to eliminate HIV-1 particles bound to the cell surface. For trypsin treatment, cells were washed with phosphate-buffered saline (PBS) and treated for 8 min at room temperature with 0.25% trypsin solution (Life Technologies, Madrid, Spain). Trypsin was stopped by addition of FCS, and cells were then washed with PBS. For intracellular staining, cells were fixed, permeabilized (Fix&Perm; Caltag, Burlingame, CA), and stained with the anti-HIV p24 antigen MAb KC57 (Coulter, Barcelona, Spain). Cells were analysed in an LSRII flow cytometer (BD, Madrid, Spain) and identified by morphological parameters and DDAO-SE staining. Quantification of HIV-1 transfer was assessed by the percentage of p24-positive T cells, using the coculture between T lymphocytes and MOLT uninfected cells as a negative control.

Comparison of cell-free and cell-associated infections in CEM-GFP cells. CEM-GFP cells were infected either with cell free virus or as a result of being cocultured with HIV-infected MOLT cells. For cell-free virus infections, 1×10^5 CEM-GFP cells were infected with 1,000 ng of an NL4-3 HIV strain (HIV_{NL4-3}) or mock infected with the same volume of medium. Coculture infections were performed at two different ratios of target to donor cells (2:1 and 9:1 CEM/MOLT ratio) and adjusted to a total of 2×10^5 cells. To differentiate effector from target cells by flow cytometry, MOLT cells were previously labeled with cell tracer DDAO-SE (Molecular Proves, Life Technologies) as described above. After extensive washes with PBS, 1.3×10^5 CEM-GFP cells were mixed with 0.6×10^5 MOLT cells at a 2:1 ratio and 1.8×10^5 CEM-GFP cells were mixed with 2×10^4 MOLT cells at a 9:1 ratio. For both ratios, cocultures with MOLT uninfected cells were performed as negative controls. Cell-free and cell-associated infections were carried out for 48 h in the presence or absence of various concentrations of AZT and TDF. Forty-eight hours postinfection, cells were recovered and infection was quantified by emission of green fluorescent protein (GFP) signal in target cells using flow cytometry in coculture infections. DDAO-SE and GFP double-positive cells, considered donor-target cell fusions, were excluded from the analysis. To quantify total GFP expression, cells were also viewed in a Nikon eclipse TE-200 microscope coupled to a charge-coupled device (CCD) Kappa camera, and the images obtained were used to quantify GFP expression. Quantification of total GFP was performed by evaluating and

quantifying the average intensity of GFP signal for every image as implemented in the Launch VisionWorks software.

HIV-independent transactivation of HIV LTR in CEM-GFP cells. Cocultures between HeLa Env+ cells (National Institute for Biological Standards and Control [NIBSC], United Kingdom) and CEM-GFP cells were performed to demonstrate unspecific replication-independent long terminal repeat (LTR)-driven expression of GFP in CEM-GFP cells [113, 114]. A total of 2×10^5 HeLa Env+ cells (also expressing Tat and Rev proteins) were seeded in a 24-well plate the day before the initiation of the coculture. After the removal of the supernatant, the coculture was initiated by the addition of 2×10^5 CEM-GFP cells in the presence or the absence of 4 μM tenofovir (TFV) in a final volume of 1 ml. Cocultures with the HeLa cell line TZM-bl (Env-) were used as negative controls. Twenty-four hours later, cells were recovered and visualized in a Nikon eclipse TE-200 microscope coupled to a CCD Kappa camera.

Quantitative real-time PCR for proviral HIV-1DNAdetection. Proviral DNA was quantified as described before [94, 95]. Briefly, cells were pelleted, supernatant was removed, and pellets were frozen at -20°C until use. Total cellular DNA was extracted using the QiaAmp DNA-extraction kit (QIAmp DNA blood minikit; Qiagen, Madrid, Spain) as recommended by the manufacturer. Quantitative amplification of LTR was performed using the following primers and probe (forward primer, 5'-GACGCAGGACTCGGCTTG-3'; reverse primer, 5'-ACTGACGCTCTCGCACCC-3'; probe, 5'-TTTGGCGTACTCACCAGTCGCCG-3', labeled with the fluorophore 6-carboxyfluorescein [FAM] and the quencher 6- carboxytetramethylrhodamine [TAMRA]). To normalize HIV copy values per cell, amplification of the cellular RNase P gene was performed using the TaqMan RNase P control reagents kit (Applied Biosystems, Roche, Barcelona, Spain). For each experiment, DNA extracted from 8E5/LAV cells (harboring one copy of integrated HIV-1 per cell) was used to build a standard curve of the proviral DNA copy number, and DNA extracted from uninfected CEM-GFP cells was used to build a standard curve of the cell number. The PCR was performed in a total volume of 50 μl using 1 × TaqMan Universal PCR master mix (Applied Biosystems), a 0.9 μM concentration of the primers, 0.25 μM probe, and 5 μl of the DNA sample. Reactions were analysed with the ABI Prism 7000 instrument using SDS 1.1 software (Applied Biosystems). For each experiment with cell-associated infections, the background of proviral DNA copy number coming from MOLT infected

cells was subtracted using a control condition in which an equal cell number of MOLT NL4-3 cells were cultured alone.

Calculation of T_x. The transmission index (T_x), designed to quantify the infection sensitivity to drugs [5], was calculated as the fraction of cells infected in the presence of drug (I_d) divided by the fraction of cells infected in the absence of drug (I). T_x depends on the multiplicity of infection (MOI) (symbolized here by the variable m), defined as a fraction of the number of infected cells by the number of target cells, and on the reduction of infection, $f(d)$, in a given drug concentration (d) as in the equation

$$T_x = \frac{I_d}{I} = \frac{1 - e^{-m/f(d)}}{1 - e^{-m}}$$

In the present study, m corresponds to the percentage of infected cells (GFP+ or p24+) in the untreated condition, which was set to roughly 4% of GFP+ cells under both cell-free and cell-associated infections. For each drug concentration tested, the T_x was calculated as the fraction of GFP+ cells in the presence of drug by the percentage of GFP+ cells in the absence of drug. T_x was equally calculated using the total HIV DNA or using the data obtained with the intracellular p24 antigen staining.

2.3.- Results

2.3.1.- Cell-to-cell transmission of HIV-1 in the absence of virus replication

We have previously shown that HIV 1 persistently infected or acutely infected T cells or dendritic cells may transfer HIV-1 particles to intracellular compartments in target CD4+ T cells [36, 51, 88]. After overnight cocultures of HIV-1_{NL4-3}-infected MOLT cells with nonstimulated primary CD4+ T lymphocytes, roughly 20% of target cells were HIV antigen positive compared to the untreated condition (**Figure 11A, black bars**). Antigen detection was resistant to the RT inhibitors AZT (4 μM) and TDF (4 μM), but was inhibited by the attachment inhibitor IgGb12 (10 μg/ml). However, at the same time point, cells remained negative of viral DNA, as measured by quantitative PCR (qPCR) (**Figure 11B, Black bars**), indicating that antigen detected in CD4+ T cells was not the product of virus replication in the target cells, but was transmitted from the infected MOLT cells. When HIV antigen-positive target cells were sorted and left for 5 days in the presence of the inhibitors, only the untreated cells remained

positive for p24 antigen staining (**Figure 11A, white bars**). Proviral DNA detection (**Figure 11B, white bars**) and p24 antigen production in the supernatant (**Figure 11C**) were only detected in untreated cells, indicating that the antiretrovirals used effectively block virus replication after cell-to-cell transmission.

In lymphoid MT-4 cells, captured virus could be detected as early as 2 h post-coculture, reached a maximum at 24 h, and was maintained for up to 48 h (**Figure 12A**). Early flow cytometry detection of intracellular virus antigen may indicate that HIV antigen in short-term cocultures does not accurately measure HIV infectivity. To confirm this hypothesis, total viral DNA in target cells was measured by qPCR. **Figure 12B** shows that despite massive intracellular p24-antigen detection, TDF and AZT clearly blocked infection even after 48 h post coculture.

2.3.2.- Cell-free and cell-associated HIV infections were equally sensitive to inhibition by reverse transcriptase inhibitors

To compare drug efficacies in cell-free and cell-associated virus transmission, CEM-GFP cells were cocultured with HIV-1_{NL4-3}-infected MOLT cells labeled with DDAO cell tracer or infected with cell-free virus (HIV-1_{NL4-3}), in the presence of various concentrations of the RT inhibitors AZT and TDF. Forty-eight hours postcoculture, infection of target cells was determined by the percentage of cells positive for GFP signal and by proviral DNA detection (**Figure 13 and 15**). As indicated in reference [5], care was taken to normalize virus input in both cell-free and cell-associated cultures, leading to roughly identical results in the percentage of GFP+ cells in the untreated condition at 48 h (**Figure 13A**). A significantly high virus concentration, roughly 5 μg/ml of p24 antigen and >25-fold higher of the commonly used virus input in drug susceptibility studies in MT-4 cells [115, 116], was required to achieve 4 to 5% GFP+ cells in cell-free infections. Under these conditions, both AZT and TDF effectively blocked virus replication with similar 50% effective concentrations (EC_{50}s) measured either by GFP signal or total viral DNA in target cells (**Table 2**).

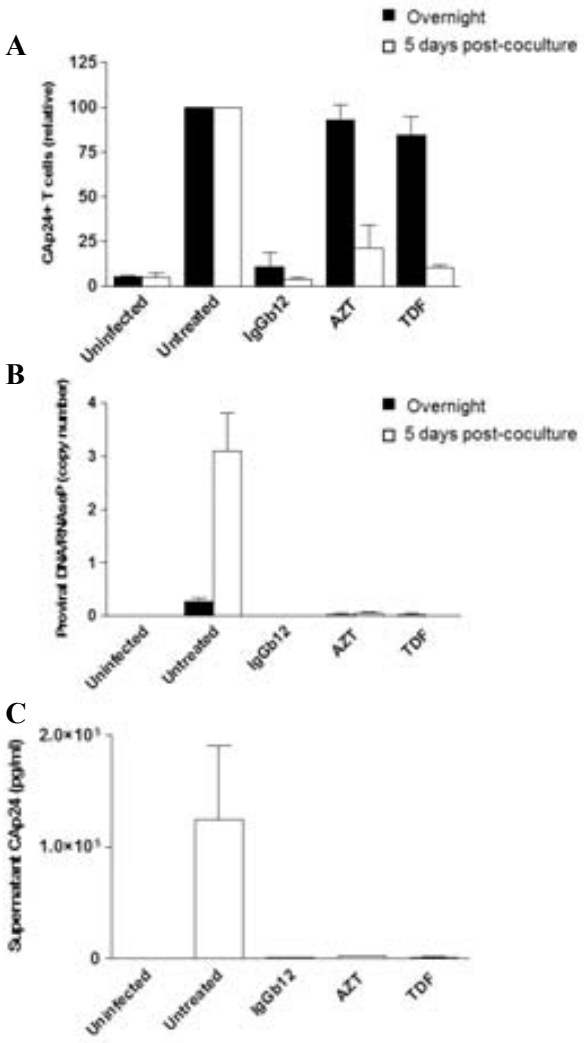


Figure 11. HIV antigen internalization in the absence of productive infection. Uninfected or HIV-1_{NL4-3}-infected MOLT cells were cocultured with primary CD4+ T lymphocytes in the presence or the absence of IgGb12 (10 µg/ml), AZT (4 µM), and tenofovir (TDF, 4 µM). After overnight coculture, target cells were sorted and left in culture during 5 days in the presence of the same compound. Quantification of transferred HIV-1 antigen transfer was assessed by the percentage of intracellular HIV-1 p24 antigen positive cells measured by flow cytometry and expressed relative to the untreated condition (**A**), and total viral DNA (proviral DNA) measured by qPCR and represented as the copy number of proviral DNA/cellular RNAse P copies (**B**) was assessed after overnight coculture (black bars) and 5 days post-coculture (white bars). **C**. Supernatant p24 antigen production was also evaluated at day 5. The data shown are the means ± standard deviations (SD) of three independent experiments.

Table 2. Similar anti-HIV activities of RTI in cell-free and cell-associated HIV-1 infections

	EC_{50} (µM)[a] of:			
	Cell-free virus		Cell-associated virus	
RTI	GFP	Proviral DNA	GFP	Proviral DNA
TDF	0,0085 ± 0,0012	0,0059 ± 0,001	0,01 ± 0,0039	0,0069 ± 0,0026
AZT	0,0086 ± 0,0054	0,0033 ± 0,0028	0,0122 ± 0,0055	0,0046 ± 0,0007

[a] The 50% effective concentration (EC_{50}) was determined by GFP and proviral DNA measurements after cell-free and cell-associated HIV_{NL4-3} infections in CEM-GFP cells cultured for 48 h to determine the dose-response curves of AZT and TDF. Considering any of the two systems employed to quantify infection, there was no significant difference in the EC_{50}s between the cell-free and cell-to-cell infections. The values shown represent the means ± standard deviations from three independent experiments.

2.3.3.- Flow cytometry evaluation of LTR-dependent GFP expression in cell-associated infections underestimates virus replication and may not accurately evaluate antiviral efficacy

Cell-to-cell culture of infected and uninfected cells may lead to Tat-dependent transactivation of the HIV LTR in the absence of virus replication [113, 114, 117]. Cocultures of HeLa Env+ cells, expressing Tat and Rev proteins, with CEM-GFP cells were performed to show HIV-1 replication-independent LTR-driven expression of GFP. Transactivation of HIV-1 LTR occurred in the absence of virus replication and could not be inhibited by TDF (**Figure 14**).

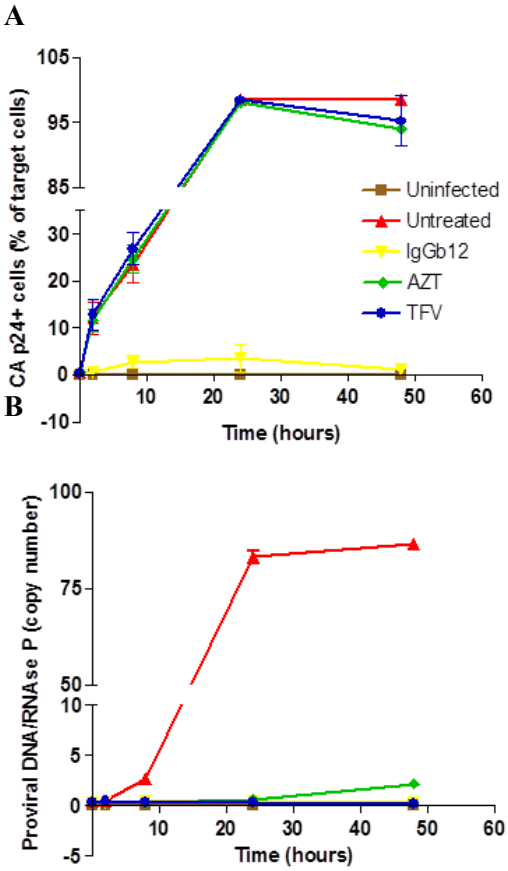


Figure 12. Virus transfer to lymphoid cells in the absence of virus replication. Uninfected or HIV-1_{NL4-3}-infected MOLT cells were cocultured with lymphoid CD4+ MT-4 cells in the presence or absence of IgGb12 (10 µg/ml), AZT (4 µM), or TDF (4 µM). Two hours, 8 h, 24 h, and 48 h post-coculture, HIV-1 antigen transfer was assessed by the percentage of intracellular p24-positive cells using the coculture between MT-4 cells and MOLT uninfected cells as a negative control (**A**). Total viral DNA (proviral DNA), represented as the copy number of proviral DNA/cellular RNAse P copies, was used to quantify infection in target cells (**B**). The data shown are the means ± SD from three independent experiments.

When comparing cell-free and cell-associated infections under conditions in which GFP expression was normalized to that of target cells by flow cytometry, the average fluorescence intensity (mean fluorescent intensity [MFI]) of the complete culture measured by fluorescence microscopy was higher in cell-associated virus than that in cell-free infections (**Figure 13B**). AZT and TDF blocked total GFP expression measured by microscopy in cell-free infections, but inhibition was only partial in cell-associated infections (48% and 25% for AZT and TDF, respectively), indicating that GFP at 48 h was a reflection of both Tat dependent transactivation and a minor component of effective viral replication. Conversely, flow cytometry data suggest a very similar inhibition in cell-free and cell-associated infections (75% versus 60% for AZT and 95% versus 84% for TDF, comparing cell-free versus cell-associated infections). This is due to the incapacity of flow cytometry to quantify GFP+ giant cell fusions, which on the other hand, may have an important component of Tat-dependent transactivation due to Env-mediated cell fusion.

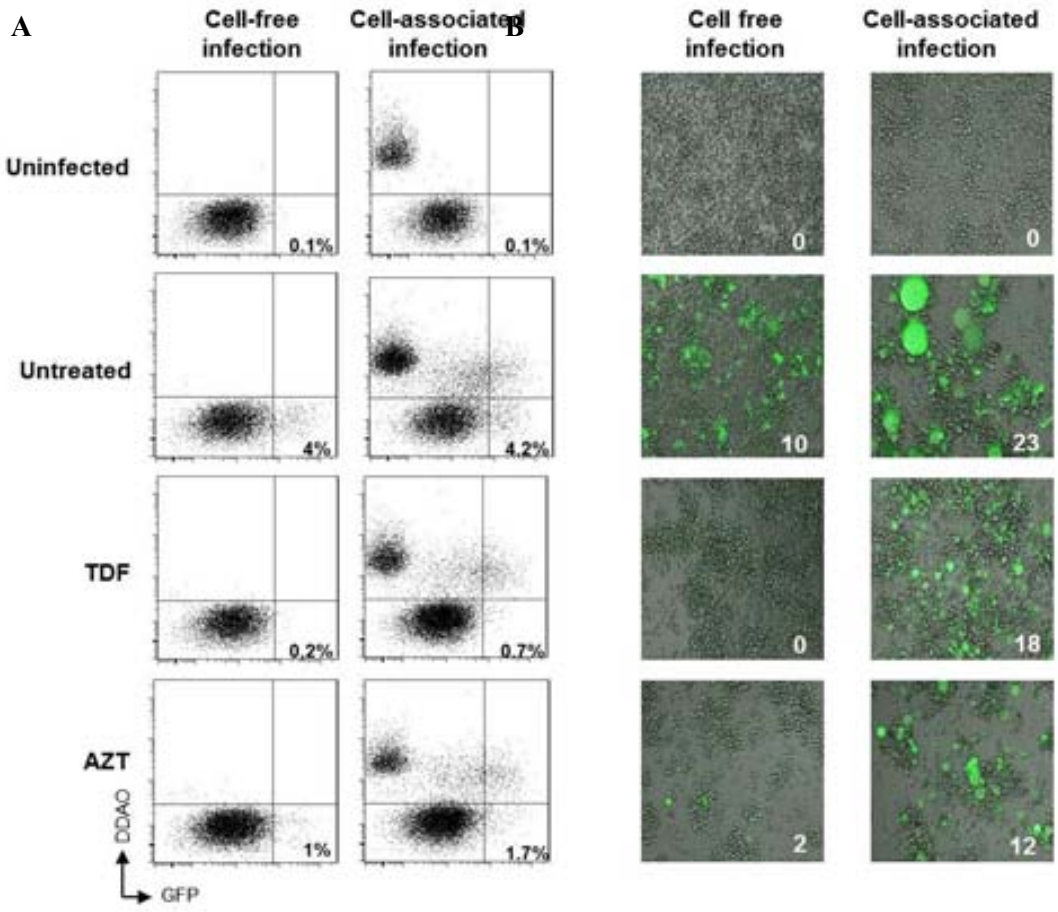


Figure 13. LTR-driven GFP did not accurately measure cell-associated infection or drug susceptibility. CEM-GFP cells were cocultured with uninfected or HIV-1_{NL4-3}-infected MOLT cells or infected by cell-free virus (HIV-1_{NL4-3}) in the presence or absence of 4 μM TDF and 4 μM AZT. Infection of target cells was determined by the percentage of cells positive for GFP signal 48 h postcoculture. (**A**) Dot plots of flow cytometry analysis corresponding to one representative experiment are shown. In the cell associated infection assay, double-positive cells were excluded from the analysis for being considered cell-cell fusions, as suggested in reference [5]. (**B**) Total LTR-driven GFP expression in the corresponding cell cultures. Values represent the average fluorescence intensity of the cell culture, as measured by green/gray pixel intensity in GFP fluorescence. Images showing the GFP fluorescence and phase-contrast overlays and the average fluorescence intensity values correspond to one representative experiment of three.

To further confirm the effect of antiretrovirals in cell-to-cell infections, total viral DNA was measured under conditions in which GFP expression was normalized to that of target cells by flow cytometry (**Figure 15A and 15C**). Total viral DNA was significantly higher in cell-associated infection; however, both AZT and TDF potently blocked total viral DNA with similar potencies (**Figure 15B and 15D**; Table 1). These results could be interpreted as cell-to-cell transmission being a more efficient mechanism of infection. However, when infectivity was normalized to total DNA by

reducing the number of infected cells in cocultures, the effects of both AZT and TDF also showed similar potencies (**Figure 15E to 15H**), indicating that total viral DNA is a reflection of the higher multiplicity of infection in cocultures that is not adequately quantified by gating GFP+ single cells, as indicated by Sigal et al. [5]. The lack of total inhibition of GFP+ cells by AZT in cell-free virus infections may be a reflection of the high virus input that was required to achieve an effect comparable to that observed in cell associated infection experiments.

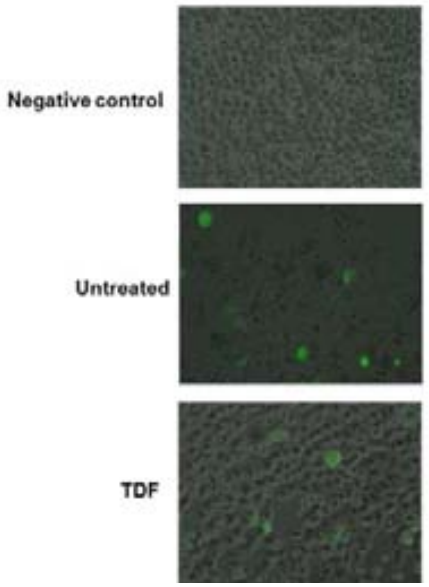


Figure 14. Transactivation of HIV-1 LTR and production of GFP occurs in the absence of virus replication. HeLa Env+ cells, also expressing Tat and Rev proteins; were cocultured with CEM-GFP cells encoding the GFP protein driven by the HIV-1 LTR in the presence or absence of 0,8 μM of TDF during 24h. As a negative control HeLa Env- cells (TZM-bl) were cocultured with CEM-GFP cells. GFP expression was evaluated by fluorescence microscopy.

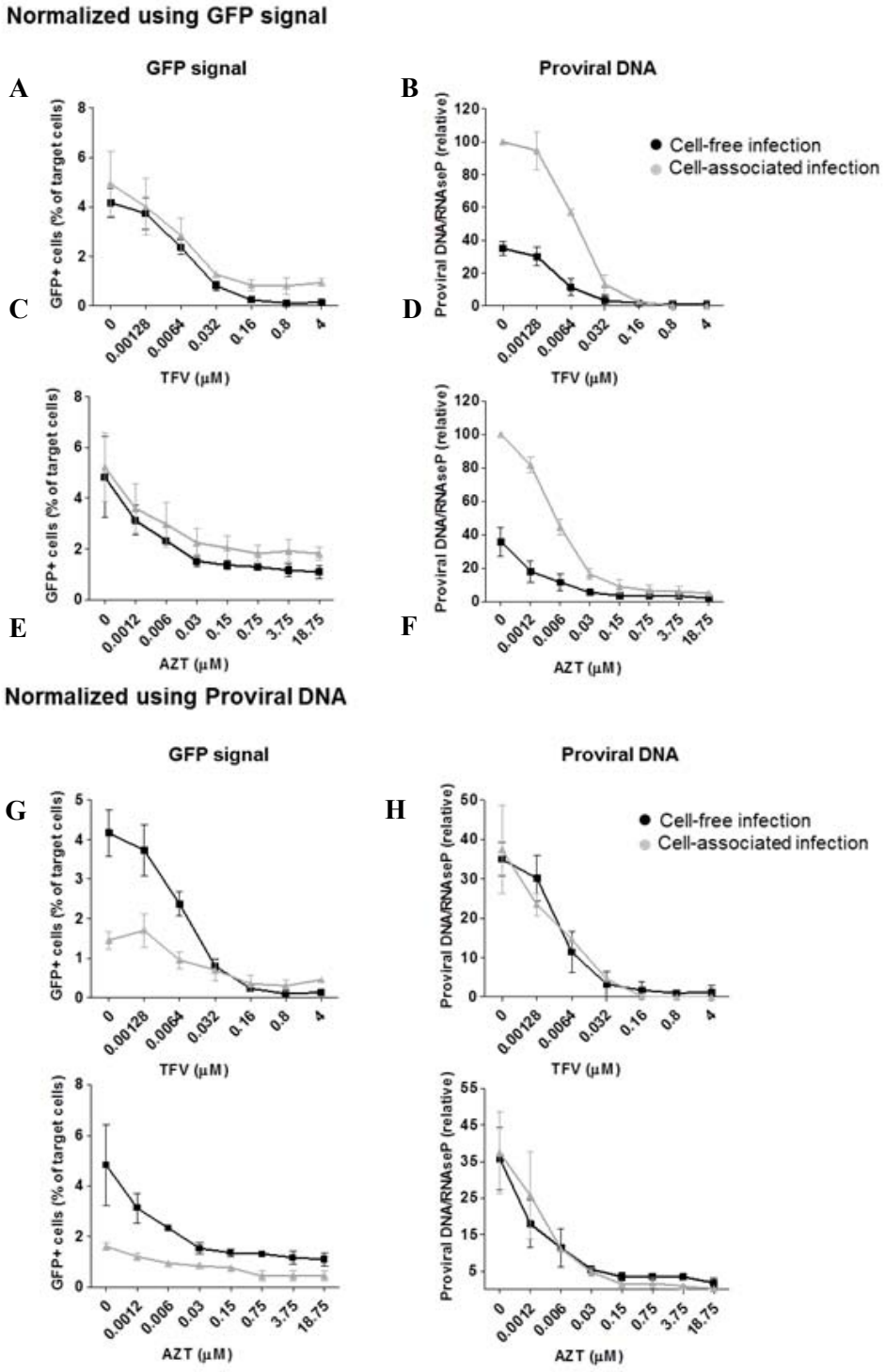


Figure 15. Potent inhibition of HIV replication in cell-free and cell-associated infection as measured by total viral DNA. CEM-GFP cells were either infected with cell-free HIV-1_{NL4-3} or cocultured with HIV-1_{NL4-3}-infected MOLT cells in the presence or the absence of serial dilutions of tenofovir (TDF) and AZT. Forty-eight hours post-coculture, infection of target cells was determined by the percentage of cells positive for GFP signal (**left panels**), as assessed by flow cytometry, or total viral DNA (**right panels**) amplified by qPCR and normalized by the cellular housekeeping gene coding for RNAse P. Cell-free (**black lines**) and cell-associated (**grey lines**) infections were normalized by the fraction of cells infected in the absence of drug using similar GFP+ values (**A, B, C, and D**) or similar proviral DNA copy number (**E, F, G, and H**). Proviral DNA data are expressed as relative to the untreated condition. Data represent the means ± SD from three independent experiments.

2.3.4.- The transmission index differs depending on how HIV infection is measured

The transmission index (T_x) represents the ratio between the fraction of infected cells in the presence of drug (I_d) divided by the fraction of cells infected in the absence of drug (I), and it is dependent on the MOI (m), defined as the product of the virus particle number and the infectivity per virus particle [5]. The calculated T_xs considering the intracellular p24 antigen staining measurements (i.e., transfer of virus) or the effective HIV infection (i.e., total viral DNA quantification) are completely different (**Figure 16A and 16B**). No inhibition of intracellular virus antigen was noted even in the presence of TDF (4 μM), but complete inhibition of replication 48 h post-coculture was observed (**Figure 13 to 16**). According to GFP expression, the potency of TDF or AZT in cell-free virus infection could not be recapitulated in cell-to-cell transmission but was clearly accomplished when total viral DNA was measured (**Figure 16C and 16D**). Thus, AZT and TDF blocked virus replication in cells that were apparently infected, as measured by FACS analysis.

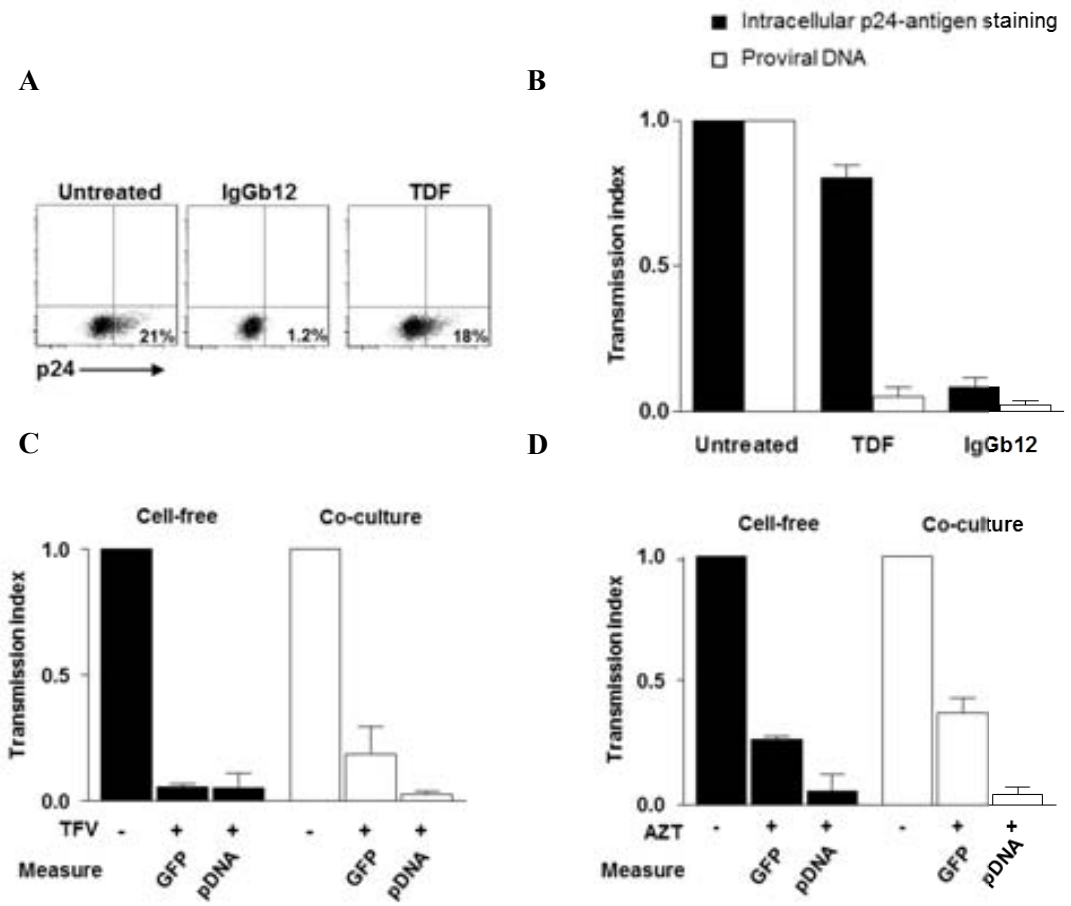


Figure 16. Transmission index (T_x) for cell-free and cell-associated infections. (**A**) Dot plots of a representative coculture between HIV 1_{NL4-3}-infected MOLT cells and primary CD4+ T cells. Values indicate the percentages of intracellular p24 antigen-positive target cells in each quadrant. (**B**) Coculture between HIV-1_{NL4-3}-infected MOLT cells and primary CD4+ T lymphocytes was performed for 48 h in the presence or absence of TDF (4 µM) and IgGb12 (10 µg/ml). Data obtained by intracellular p24 antigen staining (**black bars**) and proviral DNA quantification (**white bars**) were used to calculate the T_x [5]. The T_x was also calculated using GFP data and proviral DNA quantification after cell-free (**black bars**) and cell-associated (**white bars**) infections performed for 48 h using CEM GFP target cells in the presence (+) or absence (-) of 4 µM TDF (TFV) (**C**) or 4 µM AZT (**D**). The means ± SD from three independent experiments are shown.

2.4.- Discussion

Cell-to-cell HIV transmission has often been associated with a more efficient mechanism of infection [18, 20, 22, 118, 119]. Infected cells bind and interact with target CD4+ T cells in manners that promote virus transmission through the so-called "virological synapse" in which the interaction between CD4 and the HIV envelope glycoprotein plays a prominent role [3, 32, 82]. Effective transmission of virus is a CD4-dependent process leading to virus internalization and uncoating, reverse transcription, and subsequent steps leading to virus production from the infected cell.

Cell-to-cell transmission was recently shown as a mechanism to evade inhibition only by the anti-gp120 neutralizing antibodies, including CD4-IgG2 (Pro542), but not when other entry inhibitors were used, such as gp41-directed or cell-directed antibodies [45]. To this end, virus strains requiring addition of DEAE-dextran for cell-free but not for cell-to-cell infection were used in nonlymphoid (HeLa TZM-bl) cells. DEAE-dextran is a nonspecific polycation commonly used to enhance the association of viruses, including HIV, with target cells via relatively nonspecific charge interactions [120, 121] and may severely affect gp120 interactions with the cell surface such as to interfere with the inhibitory effect of gp120-targeting neutralizing antibodies. The results in reference [45] are in contrast to previous observations that virus attachment inhibitors, including CD4-IgG2 [51] and others [53] effectively block cell-to-cell transmission with equal potency to cell-free transmission.

We and others have shown that cell-to-cell contact may lead to the transfer of HIV particles through an endocytic internalization of fully coated virions, readily detectable in CD4+ target cells [32, 35]. However, endocytosis as a means to productive infection is a matter of ample debate. Early and recent evidence indicates that HIV may fuse with endosomal membranes to initiate a productive infection [46, 54, 89, 91], questioning the general understanding that virus entry occurs at the cell surface. Here, we recapitulate these results to demonstrate that evaluation of the presence of virus antigen in target cells at early time points does not accurately measure infectivity or drug efficacy. Conversely, detection of total viral DNA (proviral DNA) as measured by qPCR clearly demonstrates that cells remained infection negative in the presence of antiretroviral agents. Our results contrast with those of Sigal et al. [5], who evaluated intracellular antigen in cocultures of infected and uninfected cells and assumed that the presence of intracellular HIV antigen in short-term cocultures accurately measured HIV infectivity. Furthermore, to compare cell-free and cell-associated viral transmission, we used a method based on measurement of LTR-driven expression of a given marker to evaluate virus replication in cell-free virus infections, similar to that used by Sigal et al. [5]. A similar multiplicity of infection (*m*), defined as the number of GFP-positive target cells in the absence of drug, was used to compare drug efficacies in both infection systems. In line with previous observations showing that both cell-to-cell spread and cell-free viral spread were equally sensitive to entry inhibition [51, 53], drug response curves demonstrated equal efficacy of antiretrovirals, strongly indicating that cell-to-cell

transmission may not allow for ongoing virus replication in the presence of antiretroviral therapy.

Similarly to Sigal et al. we have found that antiretroviral drugs differentially blocked GFP expression, which may lead to the assumption that antiretroviral agents effectively block cell-free virus but fail to completely inhibit HIV-1 cell-to-cell spread. However, we show complete inhibition of proviral DNA in the same target cells, which, in contrast with the first assumption, points to a similar efficiency of antiretrovirals in both modes of transmission.

As previously shown [113, 114], and unlike cell-free virus infection, AZT or TDF cannot block cell-to-cell mediated Tat-dependent transactivation in the absence of replication. Evaluation of cell cultures by fluorescence microscopy demonstrated that this effect could have important consequences in cell-associated infection when syncytium formation is not impaired by the antiretroviral agent tested. Moreover, the flow cytometry analysis does not entirely evaluate infection as giant syncytia cannot be analysed. Under these conditions, the potency of antiretroviral agents is being underestimated. Thus, GFP expression may not accurately evaluate drug efficacy in cell-associated virus transmission.

The value of *m* is similar to the well-known definition of multiplicity of infection (MOI), that is, the ratio of infectious agents per target cell, a concept commonly used to normalize virus titers prior to the evaluation of drug efficacy or virus drug resistance in cell culture experiments. The convention, when comparing the efficacy of a drug against two independent virus strains (or two mechanisms of virus transfer), is to normalize the virus input and then determine the efficacy of the drug, which is commonly reported as the fold change (FC) in EC_{50}s. Assuming a similar MOI, the probability to propagate two infectious viruses (or two mechanisms of infection of the same virus) is the same, i.e., the infectivity of a virus particle does not increase by increasing the number of particles (with a higher MOI). A higher MOI raises the number of infectious events. Screening of anti-HIV agents is commonly tested at a low MOI, and plasma viral load is a predictor of treatment efficacy [122]. Sigal et al. conclude that infectivity through cell-to-cell transfer is resistant to the drug, but they have failed to evaluate both conditions (cell-free versus cell-associated infection) at similar MOI, by incorrectly assuming that each virus transferred in coculture will lead to a productive infection.

We concur with the hypothesis that multiple factors affect HIV persistence in the presence of antiretroviral treatment. However, assuming that cell-to-cell spread is permitting ongoing replication despite antiretroviral therapy is difficult to ascertain; although cell-to-cell spread has been well documented *in vitro*, its relevance in patients is still to be defined. How virus reservoirs are maintained in the presence of therapy has important clinical implications and might be the main barrier to complete clearance of HIV [107]. Therefore, data on cell to-cell spread should be taken with caution as it is crucial to correctly distinguish and measure abortive virus transfer or surrogate markers of infection (LTR-driven GFP) from effective viral replication.

2.5.- Acknowledgements

Cells, drugs, and viruses were received from the EU Programme EVA Centralised Facility for AIDS Reagents, NIBSC, United Kingdom (AVIP contract no. LSHP-CT-2004-503487) or the National Institutes of Health (AIDS Research and Reference Reagent Program). This work was supported in part by the Spanish Ministerio de Economia y Consumo (BFU2009-06958 and SAF2007-63622-02), FIPSE 360783-09 (J.A.E.), and Gala contra la SIDA 2011. M.P. and E.B. are fellows of the Spanish Fondo de Investigación Sanitaria (FIS).

Chapter 3.- The cortical actin determines different susceptibility of naïve and memory CD4+ T cells to HIV-1 cell-to-cell transmission and infection

Marc Permanyer, Eduardo Pauls, Roger Badia, José A. Esté and Ester Ballana

IrsiCaixa, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, 08916 Badalona, Spain

Submitted for publication on August 2013

Memory CD4+ T cells are preferentially infected by HIV-1 compared to naïve cells. HIV-1 fusion and entry is a dynamic process in which the cytoskeleton plays an important role by allowing virion internalization and uncoating. Here, we evaluate the role of the cortical actin in cell-to-cell transfer of virus antigens and infection of target CD4+ T cells. Using different actin remodeling compounds we demonstrate that efficiency of HIV-internalization was proportional to the actin polymerization of the target cell. Naïve (CD45RA+) and memory (CD45RA-) CD4+ T cells could be phenotypically differentiated by the degree of cortical actin density and their capacity to capture virus. Thus, the higher cortical actin density of memory CD4+ T cells was associated to increased efficiency of HIV-antigen internalization and the establishment of a productive infection. Conversely, the lower cortical actin density in naïve CD4+ T cells restricted viral antigen transfer and consequently HIV-1 infection. In conclusion, the cortical actin density differentially affects the susceptibility to HIV-1 infection in naïve and memory CD4+ T cells by modulating the efficiency of HIV antigen internalization.

3.1.- Introduction

The HIV entry process is a validated target for antiretroviral therapy [15, 17]. However, different routes and mechanisms of infection of CD4+ T cells may contribute to the establishment of HIV reservoirs and increased HIV pathogenesis [3, 82]. Resting $CD4^+$ T cells are the major reservoir of latent human immunodeficiency virus (HIV) infection and are a significant barrier to eradicating HIV because, upon stimulation, they are a source of viremia when antiretroviral therapy is interrupted [71]. Resting CD4+ T cells can be subdivided phenotypically into naïve and memory cell subsets as defined by the expression of multiple surface markers, including CD45RA, and depending on whether they have been previously exposed to a specific antigen. CD4+ memory T cells support higher levels of HIV replication than naïve CD4+ T cells, but the mechanism underlying the different susceptibility to HIV-1 infection remains unclear [74, 75, 77]. Memory resting $CD4^+$ T cells differ from naïve resting $CD4^+$ T cells in that they have a lower threshold for activation [69] and a subset of memory resting $CD4^+$ T cells express higher levels of the HIV-1 coreceptor CCR5 than do naïve resting $CD4^+$ T cells, while naïve cells express slightly higher levels of CXCR4 than memory cells [69]. However, the causes for the inherent resistance of naïve CD4+ T cells to HIV-1 infection cannot

be explained by the different expression of viral coreceptors or the degree of activation of cells [76, 77]. Furthermore, although integrated proviral infection is found in both memory and naïve resting CD4+ T cells without the need of cell activation, integration in naïve cells was lower than that in memory cells, suggesting that restriction of infection occurs at the first steps of virus life cycle [76].

Several studies have shown that the viral dependence on the actin cytoskeleton during both early processes of infection, such as fusion and entry, but also at post entry steps, are required for the establishment of infection into CD4+ T cells [98, 123-127] with a number of actin associated proteins regulating the role of cytoskeleton in viral entry [128-131]. Interestingly, a recent study found that the higher HIV-induced cortical actin dynamics in memory CD4+ T cells may promote efficient viral entry and viral DNA synthesis suggesting that phenotypic differences in the cortical actin between naïve and memory resting CD4+ T cells could account for the different cell susceptibility to HIV infection [77]. Additionally, cortical actin dynamics is also required during cell-to-cell HIV transmission by promoting the concentration of HIV antigens and its cellular receptors at the cell-cell contact zone [30]. Moreover, the uptake of HIV antigens into endocytic compartments after cell-to-cell transfer [32, 35, 36, 132] could be prevented by pharmacological disruption of the cortical actin of effector cells [19, 32, 37], suggesting that active cytoskeleton dynamics is required for the internalization process. However, the role of the cytoskeleton during cell-to-cell HIV transmission into distinct T cells subsets has not been well characterized.

Here, we show that cell-to-cell transfer of HIV-1 antigens into primary resting CD4+ T cells is dependent on the polymerization of the cortical actin. Moreover, we show that phenotypic differences in the cortical actin in naïve and memory CD4+ T cells subsets determine the degree of viral antigen transfer inducing distinct susceptibilities to HIV-1 infection.

3.2.- Materials and Methods

Ethics Statement

The work was approved by the scientific committee of Fundació IrsiCaixa. Human peripheral blood mononuclear cells were isolated from “buffy coats” of healthy blood

donors. Buffy coats were purchased anonymously from the Catalan Banc de Sang I Teixits (http://www.bancsang.net/en/index.html). The buffy coats received were totally anonymous and untraceable and the only information given was whether or not they have been tested for disease.

Cells. Peripheral blood mononuclear cells (PBMC) from healthy donors were purified by Ficoll-Hypaque sedimentation. CD4+ T lymphocytes were immediately purified (>95%) from PBMCs by negative selection using the CD4+ T cell enrichment kit (Stem Cell Technologies, Vancouver, Canada) and grown in RPMI 1640 L-Glutamine medium (Gibco, Madrid, Spain) supplemented with 10% (R10) heat inactivated fetal calf serum (FCS, Invitrogen, Madrid, Spain), 100 U/ml penicillin, and 100 µg/ml streptomycin. When needed, CD4+ T cells were stimulated with phytohemagglutinin (PHA, Sigma, Madrid, Spain) at 4 µg/ml and 6 U/ml interleukin 2 (IL-2, Roche). MOLT-4 lymphoid cell line (AIDS Reagent Program, National Institutes of Health, Bethesda, MD) was cultured in R10. Chronically HIV-1-infected MOLT-4/CCR5 cells were generated after the infection of MOLT-4 cells, with the NL4-3 X4 HIV-1($MOLT_{NL4-3}$) [133, 134]. After the infection peak, the persistently infected culture was grown and characterized for Env expression and virus production. HEK293-T cells (AIDS Reagent Program, National Institutes of Health, Bethesda, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Madrid, Spain) supplemented with 10% heat inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Cocultures of infected and uninfected cells. Non-stimulated primary CD4+ T cells were cocultured with uninfected or HIV-1 persistently infected $MOLT_{NL4-3}$ cells as previously described [36, 51, 88]. $2x10^5$ of both infected and target cells (1:1 ratio) were cocultured in the absence or presence of 10µg/ml anti-gp120 monoclonal antibody (mAb) IgGb12 (Polymun Scientific, Wien, Austria); 1µg/ml reverse transcriptase (RT) inhibitor 3'-azido-3'-deoxythymidine (AZT) or 10 µg/ml CXCR4 coreceptor antagonist AMD3100 (both from Sigma-Aldrich). Cocultures were incubated overnight at 37°C in a 96-well culture plate in a final volume of 200 µl. Primary CD4+ T cells were pretreated with serial dilutions of Latrunculin-A (max. conc.= 1µM), Phorbol 12-myristae 13-acetate (PMA, max. conc. = 15nM) or Staurosporine (max. conc.= 1µM) (all from Sigma-Aldrich) for 2 hours before coculturing for 4 hours with effector cells. Quantification of HIV-1 transfer was assessed by the percentage of CAp24-positive

CD4+ T cells, using the coculture between primary T CD4+ lymphocytes and MOLT-4 uninfected cells as a control. The use of cell lines as virus presenting cells has been extensively used by our group and others and is recognized as a useful cell culture model for cell-to-cell transmission [19, 32, 35, 36, 39, 51, 88, 100, 132].

Flow cytometry. Cells were stained with CD4, CXCR4 (12G5) or CD45RA antibodies (BD Biosciences). Intracellular staining of HIV-1 p24 antigen (CAp24) was performed as previously described [35, 36, 88, 100, 132]. Briefly, cells were fixed, permeabilized (Fix & Perm, Caltag, Burlingame, CA) and stained with the anti-HIV-CAp24 antigen mAb KC57 (Coulter, Barcelona, Spain). For F-actin staining, cells were fixed and permeabilized (Fix & Perm, Caltag, Burlingame, CA) and stained with 1µg/ml of FITC-phalloidin (Sigma) for 30 min at RT in the dark. When needed, cells were first stained with surface CD45RA for 20 min before co-staining with intracellular CAp24, phalloidin or CCF2 for fusion assays (see below). Cells were analysed in a LSRII flow cytometer (BD, Madrid, Spain) and identified by morphological parameters.

Quantification of cell-to-cell transmission. $2x10^5$ HEK293-T cells were cotransfected with 0,5 µg of HIV-1_{NL4-3} GFP (NIH AIDS Reagents Program). 48h postransfection, HEK293-T cells were cocultured overnight with primary activated CD4+ T lymphocytes. HIV-antigen transfer into naïve (CD45RA+) and memory (CD45RA-) target cells was assessed by co-staining of surface CD45RA and intracellular staining of viral capsid p24 (CAp24). After overnight coculture cells were gently shaken to break cell-cell contacts and target CD4+ T cells were carefully harvested. 4 days after target cell purification productive infection was evaluated by GFP expression and assessed using flow cytometry.

Virus-cell fusion assay. The quantification of the virus-cell membrane fusion was quantified as described before [96, 132]. Briefly, $2x10^5$ HEK293-T cells were cotransfected with 0,4 µg of both, the NL4-3 HIV provirus plasmid and a plasmid carrying the Vpr gene fused with beta-lactamase (Vpr-BlaM) (NIH AIDS Reagents Program). 48h postransfection, HEK293-T cells were cocultured overnight with primary CD4+ T lymphocytes. Cells were loaded with the CCF2-AM loading kit (Invitrogen) following the protocol provided by the manufacturer. Cells were incubated 1 h at room temperature then washed and immediately fixed. The change in emission of the cleaved CCF2 generated by the BlaM-Vpr chimera was measured by flow cytometry.

3.3.- Results

3.3.1.- The degree of actin polymerization affects cell-to-cell transfer of HIV-1 antigens

Contacts formed between HIV-1 infected and uninfected primary CD4+ T cells induce the transfer of HIV antigens into endocytic compartments in the absence of fusion or infection [32, 35, 36, 88, 132]. To evaluate the role of the cortical actin cytoskeleton of target cells during cell-to-cell HIV-1 antigen transfer, previously purified primary non-stimulated CD4+ T lymphocytes were pretreated for 2 hours with different actin remodelling compounds. After drug treatment, the degree of actin polymerization was assessed by intracellular F-actin staining (**Figure 17A and 17B**). Transient treatment of resting CD4+ T cells with latrunculin-A triggered actin depolymerisation (45% reduction in the percentage of polymerization at 1μM compared to the untreated condition). Conversely, treatment with PMA triggered actin polymerization (roughly 30% increase in the percentage of polymerization at 15nM compared to the untreated condition), consistent with a previous report [135]. Furthermore, another approach to induce actin remodelling is modulating the cofilin activity by affecting the upstream signalling using different compounds such as staurosporine [98]. However, unlike a previous report [98], we observed a slight increase of actin polymerization in staurosporine-treated cells (17% increase in the percentage of polymerization compared to the untreated condition). Drug treatment did not cause a significant change in the expression of the cellular CD4 receptor or CXCR4 coreceptor (data not shown).

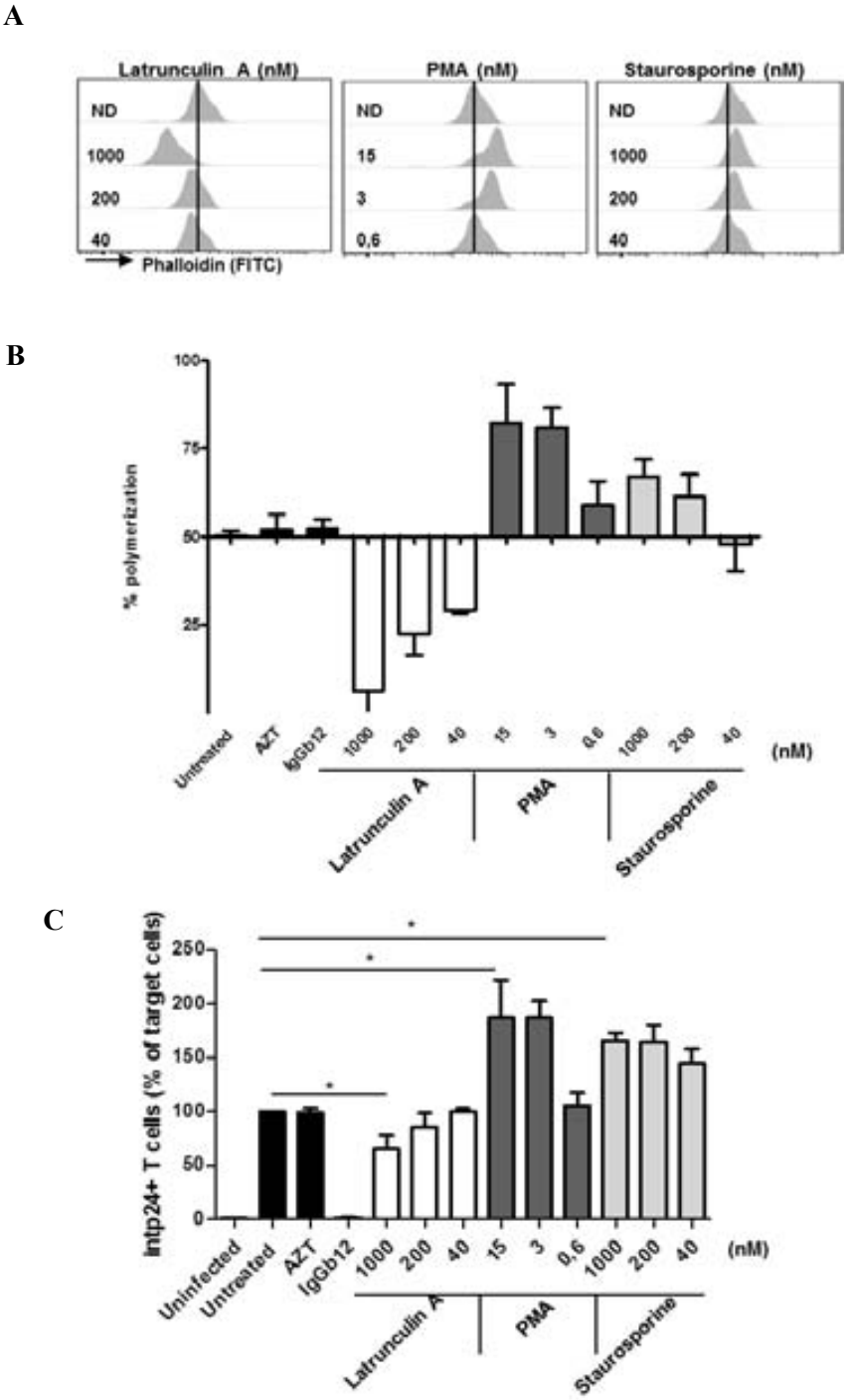


Figure 17. The cortical actin density modulates the transfer of HIV antigens during cell-to-cell contacts. Non-stimulated CD4+ T lymphocytes were pretreated with serial dilutions of latrunculin-A (max. conc.= 1 µM), PMA (max. conc. = 15 nM) or Staurosporine (max. conc.= 1 µM) for 2h. (**A**) Staining of F-actin with FITC-conjugated phalloidin was performed to assess the actin polymerization in treated cells. One representative experiment is shown. (**B**) The change in the percentage of actin polymerization normalized to the untreated condition. (**C**) Quantification of CAp24 HIV antigen transfer from infected to uninfected CD4+ T cells assessed by intracellular CAp24-antigen staining and analysed by flow cytometry. Control coculture condition were performed in the presence of IgGb12 (10 µg/ml) and AZT (1 µg/ml). The percentage of intracellular CAp24+ target cells was normalized to the untreated condition. Results are the mean ± SD of three independent experiments (*$p<0.05$).

To evaluate the effect of the cortical actin remodelling in the transfer of viral antigens, drug pretreated primary CD4+ T cells were cocultured with HIV-1_{NL4-3} persistently infected $MOLT_{NL4-3}$. After 4 hours of coculture, HIV antigen transfer to target cells was assessed by intracellular staining of CAp24 (**Figure 17C**). Compared to the untreated condition, the transfer of viral antigens to uninfected cells was clearly blocked by the neutralizing anti-gp120 mAb IgGb12 (>95% inhibition), but was not inhibited by the RT inhibitor AZT indicating a CD4-dependent transmission of high amounts of HIV-1 particles in the absence of productive infection. Furthermore, compared to the untreated condition, the transfer of viral antigens into target CD4+ T cells decreased in latrunculin A pretreated cells (40% of inhibition of CAp24+ cells at 1μM of Latrunculin A) but increased in PMA or Staurosporine pretreated cells (186% and 150% increase of CAp24+ cells, respectively at the highest concentrations tested). The lack of a more potent impact probably reflects the loss of effect over time due to the absence of the drug during the coculture. Taken together, these results demonstrate that the transmission of HIV antigens into target primary CD4+ T cells during cell-to-cell transfer is modulated by their degree of actin polymerization.

3.3.2.- Naïve and memory CD4+ T cells display distinct degree of actin polymerization

Several post-entry cellular mechanisms may explain the different susceptibility to HIV infection between naïve and memory CD4+ T cell subtypes [72, 74, 75, 77]. Because the cortical actin polymerization modulates the internalization of viral antigens during cell-to-cell contacts, we asked whether differences in cortical actin polymerization in distinct CD4+ T cell subtypes may determine different susceptibilities to infection by regulating the efficiency of viral antigen internalization. Naïve and memory CD4+ T cell subpopulations can be identified by the expression of surface CD45RA and CD45RO isoforms respectively. Thus, we performed co-staining of F-actin and surface CD45RA in primary non-stimulated CD4+ T cells to study the cortical actin polymerization of these two T cell subsets. In all donors evaluated, the intensity of the F-actin staining was higher in memory CD4+ T cells (**Figure 18A and 18B**), indicating that memory CD4+ T cells display a more polymerized actin cytoskeleton than naïve CD4+ T cells. To discard that the different susceptibility to HIV-infection in both T cell subtypes could be determined by differences in the coreceptor expression, we evaluated

the expression levels of CD4 and CXCR4 receptors in naïve and memory resting CD4+ T cells (**Figure 18C**). As expected, we did not observe significant differences in both receptors, consistent with previous reports [74, 76].

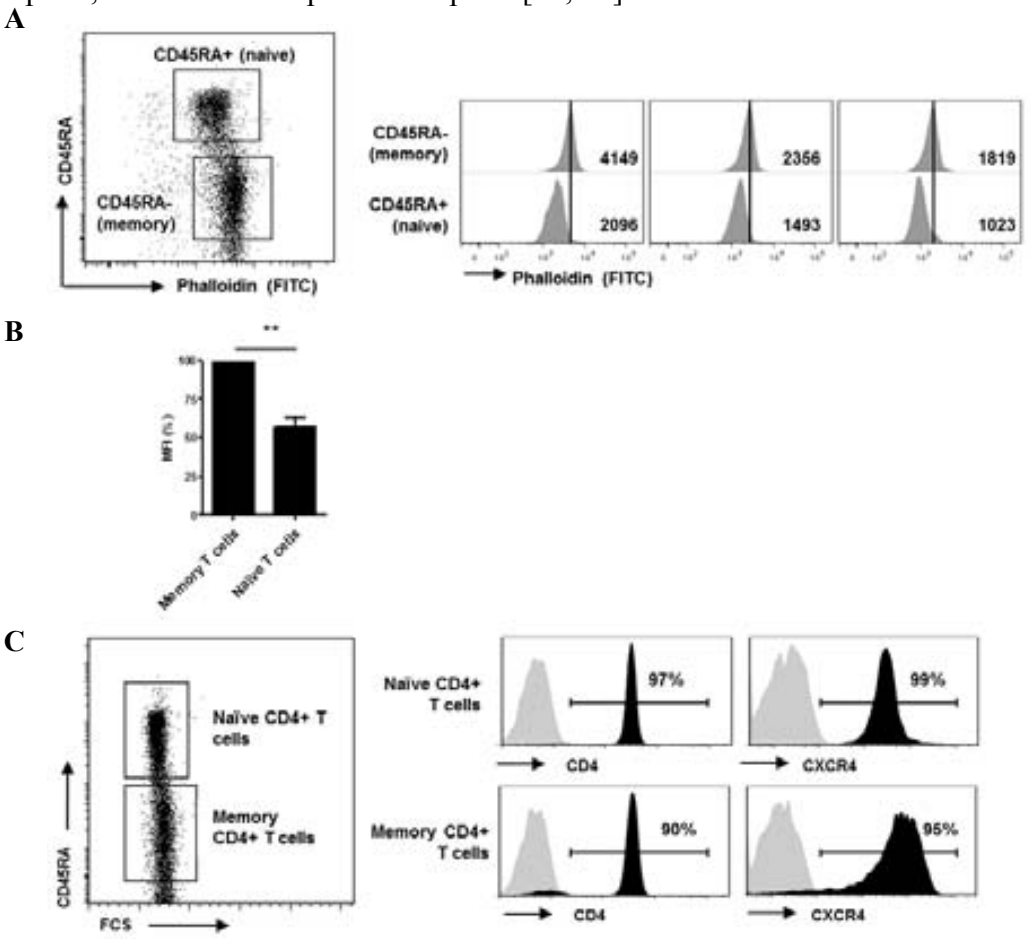


Figure 18. Distinct degree of cortical actin polymerization in naïve and memory CD4+ T cells. Resting CD4+ T cells were purified from peripheral blood by negative depletion. Surface expression of CD45RA differentiated between naïve (CD45RA+) and memory (CD45RA-) CD4+ T cells. (**A**) The F-actin in memory and naïve CD4+ T cells from 3 representative donors were evaluated by co-staining of CD45RA and FITC-labelled phalloidin and assessed by flow cytometry. (**B**) The MFI of F-actin of naïve and memory CD4+ T cells is plotted. Values are normalized to the memory T cell subset. Mean and SD of 3 different donors is shown (**p<0.005). (**C**) CD4 and CXCR4 receptor expression in naïve and memory CD4+ T cells from one representative donor is shown.

3.3.3.- Higher efficiency of HIV-1 antigen internalization into memory CD4+ T cells during cell-to-cell transfer

Given that the internalization of HIV antigens by target cells is sensitive to the degree of cortical actin polymerization, we hypothesized that the different cortical actin density in naïve and memory CD4+ T cells may induce differences in the uptake of HIV antigens during cell-to-cell transfer. To evaluate the degree of HIV antigen internalization into naïve and memory CD4+ T cell subsets, cocultures of MOLT$_{NL4-3}$ cells and primary

resting CD4+ T cells were evaluated by flow cytometry. The transfer of viral antigens to total target CD4+ T cells (**Figure 19B**) was clearly blocked by the neutralizing anti-gp120 mAb IgGb12 (>95% of inhibition compared with the untreated condition), but was not inhibited by the RT inhibitor AZT (23% of p24+ cells). As previously described [35, 36, 132], these results indicated a CD4-dependent transmission of high amounts of HIV-1 particles from infected to uninfected cells in the absence of fusion or infection. On the other hand, in all conditions the uptake of HIV particles by memory CD4+ T cells increased roughly 3-fold in untreated or AZT-treated target CD4+ T cells (10% and 35% of p24+ cells respectively), compared to naïve target CD4+ T cells (**Figure 19C**), consistent with a previous report [39]. These results indicate that phenotypic differences between naïve and memory CD4+ T cells establish different affinities for HIV antigens during cell-to-cell viral antigen internalization, suggesting that the susceptibility to HIV infection may be determined during early internalization processes that may be related to cortical acting polimerization.

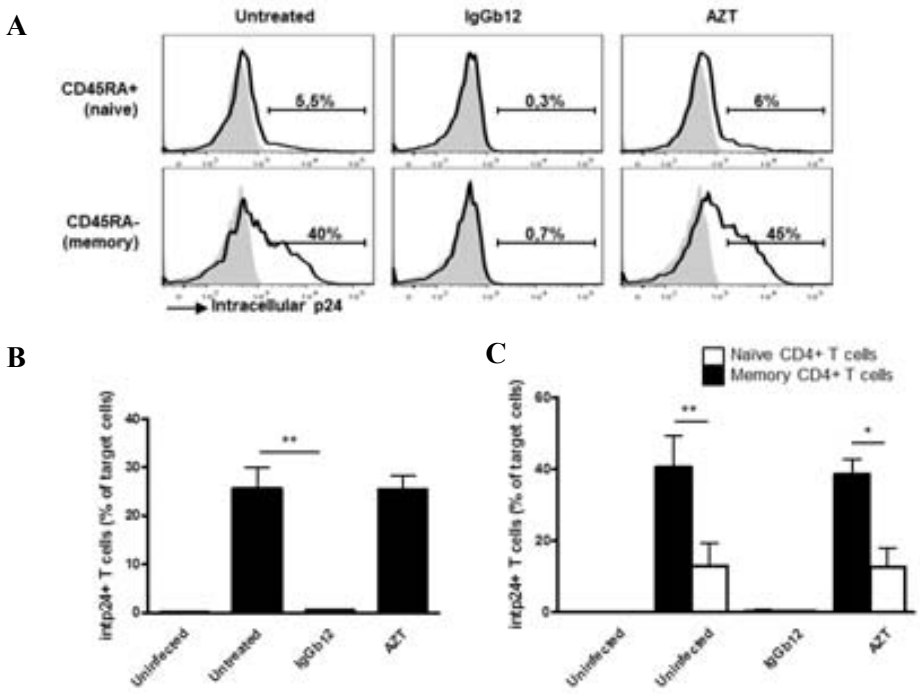


Figure 19. Different HIV-antigen internalization into naïve and memory CD4+ T cells. Uninfected or infected $MOLT_{NL4-3}$ cells were co cultured overnight with non-stimulated primary CD 4+ T lymphocytes in the presence or the absence of the anti-HIV-1gp120 mAb IgGb12 (10 µg/ml) or the RT inhibitor AZT (1 µg/ml). Quantification of CAp 24 HIV antigen transfer from infected to naïve and memory CD4+ T cells was assessed by co-staining of CD45RA and intracellular CAp24-antigen staining and analysed by flow cytometry. (**A**) One representative experiment is shown. (**B**) Percentage of intracellular CAp24+ into total target CD4+ T cells. (**C**) Percentage of intracellular CAp24+ into naïve (CD45RA+) and memory (CD45RA-) CD4+ T cells. Mean and SD of three independent experiments is shown (**p<0.005, *p<0.05).

3.3.4.- Higher efficiency of cell-to-cell transmission of HIV-1 into memory CD4+ T cells

The susceptibility to HIV infection of naïve and memory CD4+ T cells has not been previously evaluated in the context of cell-to-cell transmission which is considered to propagate HIV infection more efficiently than cell-free virus spread [18, 53] through endocytic internalization of viral antigens in the absence of virus-cell fusion or infection [32, 35, 36, 88, 132]. To assess the cell-associated transmission of HIV antigens into naïve and memory CD4+ T cells, activated primary CD4+ T cells were cocultured with HEK293T cells previously transfected with HIV-1_{NL4-3} GFP. This system allowed us to evaluate in parallel the non-productive transfer of viral antigens and the subsequent establishment of a productive infection. HIV-antigen transfer into naïve (CD45RA+) and memory (CD45RA-) target cells was assessed by intracellular staining of viral capsid p24 (CAp24) after overnight coculture (**Figure 20A**) and productive infection was evaluated by GFP expression 4 days after target cell purification (**Figure 20B**). As shown before, the transfer of viral antigens to uninfected cells was clearly blocked by the neutralizing anti-gp120 mAb IgGb12 (90% and 95% inhibition compared to the untreated condition in memory and naïve CD4+ cells respectively), but was not inhibited by the RT inhibitor AZT. After target cell purification, cells were left in culture in the presence of the same compounds. 4 days after culturing, infection of memory CD4+ T cells increased roughly 3-fold compared to naïve CD4+ T cells (6% and 2% of GFP+ cells in memory and naïve CD4+ T cells respectively) (**Figure 20B**) consistent with previous results [73-75]. As expected, IgGb12 and AZT effectively block virus replication after cell-to-cell transmission [100]. Altogether, these results indicate that when cells are permissive to infection, virus replication is in concordance to the amount of virus transferred during the coculture phase suggesting that the susceptibility to HIV infection may be determined during early internalization processes even before viral entry.

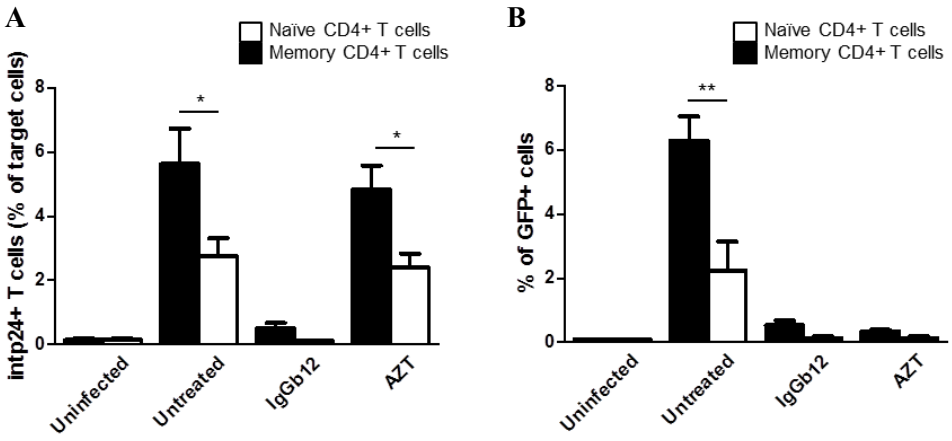


Figure 20. Higher efficiency of cell-to-cell HIV-1 transmission into memory CD4+ T cells. HEK293-T cells cotransfected with HIV-1_{NL4-3} GFP plasmid were cocultured with primary activated CD4+ T cells in the presence or the absence of the anti-HIV-1gp120 mAb IgGb12 (10 μg/ml) and the RT inhibitor AZT (1 μg/ml). After overnight coculture target cells were carefully harvested after gently shaken and cultured for 4 days in the same inhibitors. (**A**) Percentage of intracellular CAp24+ into naïve (CD45RA+) and memory (CD45RA-) CD4+ T cells after overnight cocultures. (**B**) Percentage of naïve (CD45RA+) and memory (CD45RA-) GFP+ cells 4 days after purification of target cells. Mean and SD of three independent experiments is shown (**p<0.005, *p<0.05).

3.3.5.- Viral entry is not restricted in any of both T cell subsets

Discrepant results have been reported regarding viral fusion of HIV-1 into naïve and memory T cell subsets [76, 77]. One previous report showed that naïve T cells were restricted at viral fusion [76], while the other study found only slightly diminished viral fusion in naïve T cells in one of two assays [77]. To find out whether HIV antigens are prevented from entering a subset of CD4+ target cells after cell-to-cell transfer, we evaluated the efficiency of viral entry in both T cell subtypes using the Vpr-β-lactamase-based entry assay [96]. HIV_{NL4-3} transfected *Vpr*-BlaM+ HEK293-T cells were cocultured with primary resting CD4+ T cells and fusion was measured in naïve (CD45RA+) and memory (CD45RA-) T cell subtypes by detection of the enzymatic cleavage of CCF2 dye using flow cytometry (**Figure 21A**). We found that viral entry into naïve CD4+ T cells was reduced roughly 25% compared to memory CD4+ T cells but the difference was not statistically significant (**Figure 21B**). Viral fusion into naïve CD4+ T cells was significantly inhibited by IgGb12 (95% reduction). Surprisingly, IgGb12 did not block viral fusion into memory CD4+ T cells as efficiently as into naïve CD4+ T cells (70% of inhibition compared to the untreated condition) (**Figure 21B**). As expected, cleavage of CCF2 was not prevented by AZT. Taken together, these results

indicate that after cell-to-cell transfer, viral entry is not restricted in any of the CD4+ T cells subtypes.

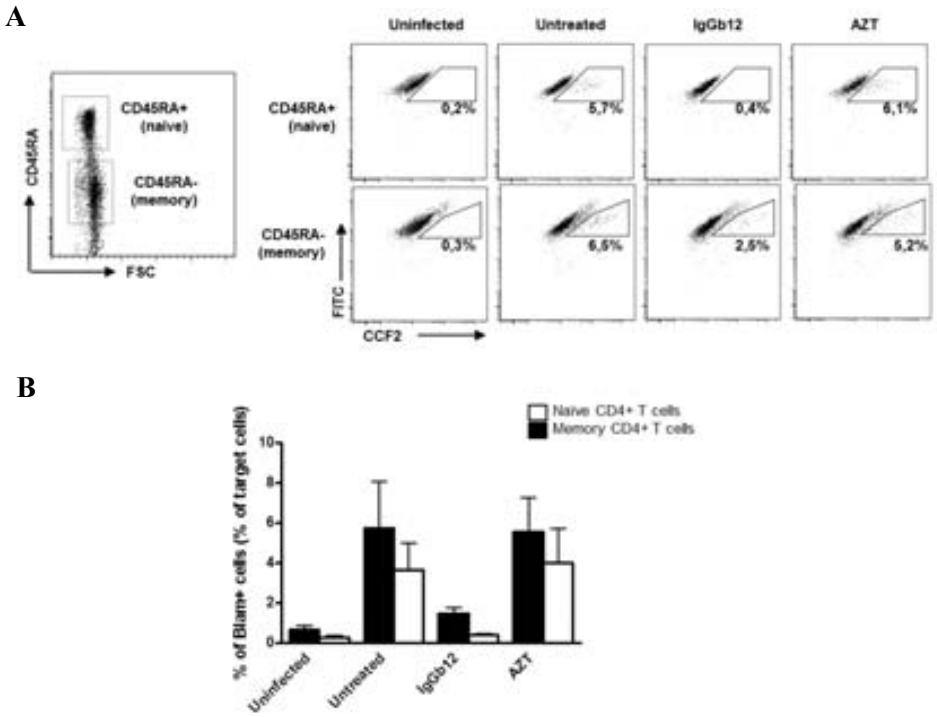


Figure 2 1. S imilar le vels o f viral fusion i nto naïve a nd memory CD 4+ T c ells. HEK293-T cel ls cotransfected with pNL4-3 and BlaM-Vpr plasmids were cocultured with primary resting CD4+ T cells in the presence or the absence of the anti-HIV-1gp120 mAb IgGb12 (10 µg/ml) and the RT inhibitor AZT (1 µg/ml). Viral fusion was assessed by flow cytometry by measuring the percentage of CCF2-cleaved naïve (CD45RA+) a nd m emory (CD4 5RA-) targ et CD4 T c ells. (**A**) Dot plots o f CCF2-loaded cells (FITC-labelled) versus CCF2-cleaved cells (Pacific blue-labelled) of a representative experiment are shown. (**B**) Percentage o f CCF 2-cleaved tar get cells normalized to m emory CD4 + T c ells in u ntreated c ondition. Mean and SD of two independent experiments is shown.

3.4.- Discussion

Actin pol ymerization but also other fa ctors ha ve be en pr oposed to contribute to the different HIV susceptibility of naïve and memory T cells, especially expression of cell surface proteins, such as viral coreceptors, or the degree of activation of cells [76-78]. Here, w e show that c ortical actin de nsity pla ys a pr ominent role in determining susceptibility to HIV-antigen c apture a nd inf ection, mapping the restriction at early steps of viral life cycle after virus-cell fusion.

Our re sults are c onsistent with previous re ports showing that the differential susceptibility in naïve and memor y C D4+ T cells can a lready b e de tected during the

initial stages of viral infection such as viral entry or DNA synthesis [72, 73, 76, 77]. Interestingly, differences in the cortical actin between naïve and memory CD4+ T cells affecting viral DNA synthesis have been recently reported in cell-free virus infections [76]. Unlike cell-free virus infection, our short-term coculture model between HIV-infected and non-stimulated primary CD4+ T cells maximise the cell-to-cell endocytic antigen transfer, which might determine differences in the infection outcome. There exist several evidences of the importance of viral entry route in relation to cytoskeleton remodelling and establishment of HIV infection. Viral entry via endocytosis may allow viruses to overcome the restriction of a static cortical actin or to evade antibody neutralization [46]. Moreover, it has been shown that cell-free and cell-to-cell HIV-1 infections were not equally sensitive to the actin inhibitor cytochalasin D [32] and, neither resting nor activated T cells are equally susceptible to infection by VSV-G-mediated endocytosis [55]. We have previously demonstrate that transferred HIV particles resurface to the outer cell membrane of resting CD4+ T cells, suggesting that endocytic uptake may serve as an itinerant virus reservoir capable of inducing trans-infection of cells after the release of HIV particles to the extracellular environment, but being unable to establish productive infection [36, 82, 132]. Thus, the infection system, the cell type or the activation state of the target cell may also condition the entry route [82], which simultaneously may impose different cytoskeleton requirements.

Reduced levels of virus-cell fusion in naïve CD4+ T cells were suggested to be responsible for the restriction in this subset of T cells [76], an observation that is in clear contrast with our results showing no significant differences in virus-cell fusion between memory and naïve T cells. Probably, the discrepancy between this report and our observations may arise from the different infection systems used. The higher efficiency of cell-to-cell HIV transmission compared to cell-free virus infection [3, 18, 53] may allow to overcome restrictions observed during cell-free virus infection and thus may minimise the qualitative differences between both T cell subtypes. Taken together, our results are in accordance to the findings reported in by Wang et al., [77]. Both conclude that cortical actin polymerization determines the susceptibility to infection during the steps prior to viral entry, through modulating the uptake of HIV-antigens during cell-to-cell transfer. It cannot be discarded that cytoskeleton remodelling may have an effect over other cellular processes involved in the viral infection cycle such as reverse transcription or budding as suggested by others [37, 77]. Similarly to cell-free virus

infection [123-126], during cell-to-cell transfer there is an *Env*-induced actin-dependent HIV-receptor clustering at the cell-cell interface [30]. Thus, the different degree of actin polymerization in naïve and memory CD4+ T cells may induce distinct cellular receptor recruitment in both T cell subtypes, which in turn, may affect the efficiency of HIV-antigen internalization [136], in both cell-free and cell-to-cell virus infection.

Eradication of HIV-1 with antiretroviral therapy is not possible due to the persistence of long-lived, latently infected resting memory CD4+ T cells. The demonstration of a role of the cortical actin in HIV cell-to-cell transfer and infection of memory and naive CD4+ T cells may provide a mechanistic understanding of viral infection and pathogenesis. The higher HIV antigen capture and the broader pattern of migration of memory CD4+ T cells [69] may contribute to a more efficient dissemination of infection that in turn are coupled with additional changes in the cortical actin during the migration to of these HIV-antigen loaded primary CD4+ T cells would also favour infection [137]. Thus, defining the restriction imposed by the cortical actin during HIV infection in different T cell subtypes and how the virus overcomes this constraint specifically in naturally resistant resting CD4+ T cells may be relevant for understanding the pathogenesis of HIV and for the development of new drug therapies.

3.5.- Acknowledgements

We thank the National Institutes of Health (AIDS Research and Reference Reagent Program) and the EU Programme EVA Centralised Facility for AIDS Reagents, NIBSC, UK for reagents. This work was supported in part by the Spanish Ministery of Economy and Competitiveness (MINECO), project BFU2012-06958 and Gala Against AIDS. MP and EP are research fellows from MINECO and Fondo de Investigación Sanitaria (FIS). The authors declare no competing interests.

DISCUSSION & PERSPECTIVES

To better understand the infectious pathway during cell-to-cell transmission of HIV-1, we evaluated the fate of HIV particles captured by target CD4+ T cells after cell-to-cell transfer. Consistent with earlier investigations showing transferred HIV-1 virions into trypsin-resistant compartments, positive for clathrin and the early endosomal marker EEA1 but negative for the marker of lysosomal degradation Lamp-1 [35, 36], we have found that cellular contacts between infected lymphoid cells and primary CD4+ T lymphocytes triggered CD4-dependent transmission of high amounts of HIV-1 particles into trypsin resistant compartments in the absence of virus replication. Together with other recent reports [19, 32, 46], these results indicate that virus internalization during cell-to-cell transfer of HIV-1 occurs through a clathrin-dependent endocytic pathway. This results showing endocytosis to be the mode of HIV entry are in apparent contrast to previous reports that concluded that the was no overlap between HIV-1 antigen and early endosomes [30] and failed to observe endocytic uptake of HIV-1 within intracellular compartments using electron microscopy or electron tomography [53] suggesting direct viral fusion with the target cell plasma membrane.

Discrepancies in the mode of HIV entry could be the result of differences in the variety of culture models and cell culture conditions, including the ratio of infected to uninfected T cells, the chronicity of the infection of the effector cells, the type of cell or cell line used, the degree of activation of target cells or the rate of HIV gp120 engagement with cell surface receptors. The target cell type used (laboratory adapted cell lines or different types of primary cells) may influence the virus binding to the cell surface, regulated by several attachment factors differentially expressed in different cell types [57, 138-140] which in turn may determine the infectivity of the target cell. Moreover, the variable delay between the virus uptake and fusion in different cell types may prevent to observe virus-cell fusion at the cell membrane by premature removing of fusogenic viruses from the cell surface. Furthermore, the degree of activation of target cells determine the cell fusogenicity; compared to non-stimulated cells, activated target cells may be more permissive for HIV-fusion [141], and so endocytosis may be less dominant. Thus, the degree of activation of target cell may not only determine the susceptibility to infection but also the virus entry pathway [55].

Thus, a combination of factors such as the cell type specific entry receptor levels, Env fusion kinetics and endocytosis rates can determine whether fusion occurs at the plasma membrane or from endosomes (**Figure 22**). Rapid fusion kinetics at the cell membrane promoted by mature viral particles with envelope glycoproteins with high affinity for receptors and high degree of receptor or coreceptor expression and/or cell activation, might favor virus-cell membrane fusion, leading to a productive infection. Conversely, transmission of fusion-inefficient immature viral particles, low CD4 or coreceptor expression and/or low cellular activation drive low fusion kinetics, leading to the accumulation of virus particles at the cell surface and subsequently the engulfment of virus particles by the target cell, from which some virus particles will proceed to lysosomes, others will fuse with the endosome membrane, and some might be recycled back to the cell surface. Similarly, inhibition of viral fusion without blocking the transfer of viral antigens using entry inhibitors such as AMD3100 or C-34 induce the accumulation of large amounts of viral particles in a "non-fusogenic" form into the cell membrane increasing its absorption through an endocytic mechanism [35, 36].

Finally, the method of detection of viral transfer may also be important. The HIV-1 Gag-antigen staining allows easily quantification of viral antigen transfer by flow cytometry and localization using confocal microscopy. Moreover, intracellular HIV Gag-antigen detection can only be block by agents that prevent virus attachment, but is resistant to other HIV entry inhibitors suggesting that intracellular HIV antigen detection in target cells at early time points measures passive transfer of viral antigens in the absence of virus-cell fusion or replication. In contrast to other reports showing a significant role for the endocytic compartment in promoting viral entry and infection [46, 54, 142], we found that endocytosed viral particles could not initiate a productive infection from within endosomal compartments of primary CD4+ T cells. Instead, transferred viruses required to resurface and reach the extracellular environment and reengage CD4 leading to virus-cell fusion and replication, a condition that could only be achieved in the absence on the attachment inhibitor IgGb12. Several possibilities may explain these discrepancies. The impaired fusogenic capacity of newly internalized HIV particles may lead to the dissociation of HIV gp120-CD4 receptor allowing the recycling of viral particles to the extracellular milieu. A similar effect could be achieved by treatment with entry inhibitors [143]. The degree of activation of target cell may be another important variable. Even though HIV fusion and entry of transferred HIV

particles may not be not impaired, post-entry restrictions may block infection of non-stimulated T cells [55, 98, 137]. Upon T cell activation and in the absence of HIV entry inhibitors, the recycled virus might induce a productive infection when conditions of high fusion kinetics are present. In contrast to previous observations [54], we found that inhibition of the GTPase dynamin, an essential protein involved in the clathrin-dependent coated-vesicle formation, did not block cell-to-cell virus transfer, fusion or productive infection into primary CD4+ T cells. Differences between our and their results could come from the target cells used; while Miyauchi et al. [55] conducted their studies using TZM-bl cells derived from the transformed epithelial HeLa cell line and consequently having very active endocytic machinery, we used non-stimulated primary CD4+ T cells, the main target for HIV infection. Moreover, as previously observed using electron microcopy, dynasore did not prevent the accumulation of coated pit intermediates anchored into the cell membrane [101] suggesting that dynamin may not prevent the initial formation of the coated vesicles. In fact, using confocal microscopy we showed that dynasore could not prevent even the recruitment of the dynamin into the newly-formed coated pits. Thus, HIV-antigen internalization into primary CD4+ T cells after cell-to-cell transfer may occur in early endocytic compartments. In agreement with our results, VSV-G endocytic-mediated entry and wild-type HIV envelope-mediated entry were not equally sensitive to inhibition by the dynamin-dependent endosome-scission inhibitor dynasore [55], indicating that both entry pathways may have different requirements. Moreover, if dynamin is involved in both fusion and endocytosis [91], it seems difficult that both effects can be assessed and quantified separately because one can not be inhibited without affecting the other. In the absence of endosome maturation these vesicles could act as reservoirs capable of inducing trans-infection of cells after the release of HIV particles to the extracellular environment leading to infection of bystander CD4+ T cells.

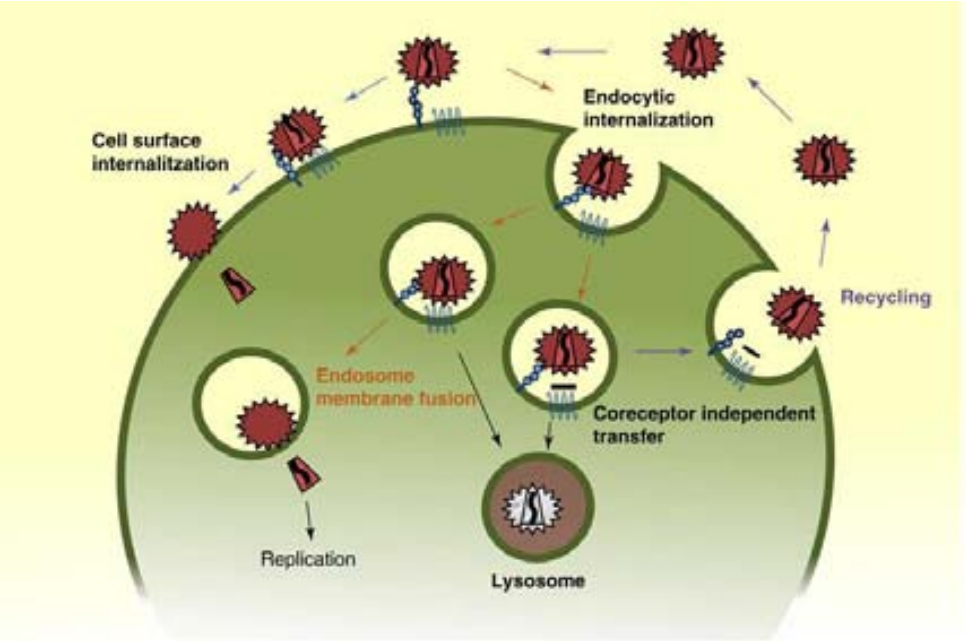


Figure 22. Putative HIV entry pathways. After binding of HIV to the CD4 receptor, the virus can enter target cells through three non-exclusive pathways. The first pathway is the canonical or direct fusion of HIV particles with the plasma membrane (cell surface internalization). In the second pathway, endosomal internalization establishes HIV fusion with an endosome membrane following an endocytic uptake of virus particles, or this could lead to endosome maturation and lysis of the virus particle. In the third pathway, coreceptor engagement might lead to virus membrane and endosome membrane fusion and release of the virion into the cytosol; alternatively, in the absence of the appropriate coreceptor or coreceptor independent transfer, viruses might be recycled back to the extracellular medium as infectious particles capable of mediating a productive infection.

We have found that infection can only be abrogated if cells are cultured in the presence of IgGb12, an inhibitor of virus attachment to CD4+ T cells. Therefore, cells carrying endocytosed virus became infected only if virus was discharged to the cell supernatant and allowed to re-infect cells. This mechanism would present several advantages for the virus; endocytosed viruses may hide without leaving any evidence of their presence or may avoid processing and exposing viral antigens by the immune system delaying the onset of an antiviral response. Furthermore, cells containing endocytosed virus may act as carriers of HIV to tissues and organs where drugs could not reach, free their cargo and become infected or infect bystander CD4+ T cells. In summary, endocytosis may not be the primary mode of entry leading to virus replication but it may serve as a reservoir that transiently protects virus from a negative environment.

In the context of viral pathogenesis, direct cell-to-cell transmission may confer a number of advantages compared to cell-free infection. Firstly, cell-to-cell spread increases infection kinetics by directing virus assembly and budding to sites of cell-cell contact obviating the rate-limiting step of extracellular diffusion that is required for cell-

free virus infection. Furthermore, polarizing virus towards sites of cell-to-cell contact increases the number of potentially productive transmission events. Although it is well known that cell-cell contacts spreads HIV infection more efficiently than cell-free virus [18, 20, 53, 144], discrepant results have been reported regarding the sensitivity to neutralization of both infection mechanisms by humoral immunity [19, 45, 52, 53] or by antiretrovirals [5, 145]. The different sensitivity to inhibition of both transmission modes may be explained by the distinct multiplicity of infection [5, 21, 22, 145], due to qualitative differences in the mode of viral transmission [32, 46] or by providing a relatively protected environment at the cell-cell contact zone which could physically exclude antibodies from gaining access to virions [33, 34, 53].Diminished drug sensitivities during *in vivo* cell-to-cell transmission may have adverse consequences for the immune system and could potentially contribute to viral persistence leading to therapy failure.

Studies performed using patient neutralizing sera demonstrated that the immature conformation of cell-cell transferred HIV antigens allowed viruses to evade antibody neutralization [19, 32, 46], establishing mechanistic differences between cell-associated and cell-free virus infection systems. Consistent with these results, cell-to-cell transmission was shown to evade inhibition by gp120 specific agents including CD4-IgG2 (Pro542), but not when other entry inhibitors were used, such as gp41-directed or cell-directed antibodies [45]. In this study, the DEAE-Dextran was used to enhance cell-free virus association with the non-lymphoid TZM-bl cell line. This nonspecific polycation enhances adsorption by receptor and envelope-independent mechanisms [146] and could therefore have masked the effect of the gp120-targeting neutralizing agents. In contrast, previous observations indicated that cell-to-cell transmission was completely blocked by CD4-IgG2 and other anti-gp120 or gp41 antibodies [51, 52] suggesting that HIV infection between T cells is transmitted by a neutralization-sensitive mechanism [52, 53].

Discrepant results could arise from the diversity of experimental approaches that have been used. Infection in both systems has been equalized and quantified using a wide range of parameters; from very early infection events such as fusion [19, 46] or *de novo*-synthetized DNA [53] until late infection events such as GFP-expression [32] or CAp24 production [5]. Moreover, different assays have been used during cell-free virus infections to closely resemble the conditions of cell-to-cell transmission, such as virus-

permeable transwells [53] or maintaining cocultures in agitation [18]. However, cell-associated transmission is much more cytopathic than cell-free virus transmission, which is hardly taken into account when comparing both infection systems.

We and others have demonstrated that coculture of HIV-1 infected cells with CD4+ T cells, the model used by Sigal et al., leads to detection of high amounts of enveloped virios in clathrin-coated endosomes [32, 35, 36, 132] that may not represent a productive infection. Capture of viral antigens may reach its maximum after 6 hours of coculture and persists for 48h, or as long as target cells are cultured with infected cells [36]. In permissive cells, the capture of viral antigens may be confounded with *de novo* virus production. Cell to cell HIV transfer is blocked by virus attachment inhibitors, but is resistant to coreceptor antagonists, fusion inhibitors and importantly, agents targeting the reverse transcriptase, or subsequent steps in the HIV replication cycle. However, despite massive antigen detection in target cells after cocultures, antiretrovirals efficiently inhibited the detection of proviral DNA and the production of viral antigens in the supernatant indicating that infection was clearly blocked. Consequently, early antigen detection by intracellular staining of HIV CAp24 antigen in target lymphoid cells after short-term cocultures, similar used by Sigal [5], does not accurately measure infectivity and consequently does not correctly measure drug efficacy [100].

In addition, to compare the inhibitory efficiency of antiretrovirals in cell-free and cell-to-cell transmission, we used a system based on the LTR-driven GFP expression in target cells as a measure of infection [147]. However, under this conditions, we found that total GFP expression evaluated by fluorescence microscopy did not accurately measure cell-associated infection or drug susceptibility due to the Tat-dependent LTR transactivation in the absence of virus replication caused by the formation of syncytia and not prevented by the antiretroviral agents [113, 114]. Consequently, compared to cell-free virus infection, monitoring cell-associated infection and drug susceptibility using reporter cell lines can lead to an overestimation of the HIV infection and therefore to an underestimation of the efficacy of the antiretrovirals. Thus, similar to results of Sigal et al., this replication-independent GFP expression prevented to observe a complete inhibition of cell-associated infection.

Consistent with other results [51-53], evaluation by flow cytometry which allowed to leave out syncytia showed that cell-free and cell-associated infections were equally sensitive to inhibition, with similar dose response curves and EC50 values, recapitulating the efficacy of antiretrovirals in cell-free virus infections and discarding cell-to-cell transmission as a mechanism allowing residual viral replication during antiretroviral therapy [148]. Potent inhibition of HIV replication in cell-free and cell-associated infections by AZT and TDF was further confirmed by total viral DNA detection. Interestingly, when infection was normalized in both infection systems by the GFP signal, total viral DNA was higher in cell-associated infection which may indicate that cell-associated infection is a more efficient mechanism of HIV spread [20, 53] which may allow multiple HIV proviruses to be simultaneously cotransmitted [21, 22]. However, higher amounts of total viral DNA in cell-associated transmission may be indicative of a higher multiplicity of infection which may invalidate the normalization done by gating single GFP+ cells, as indicated by Sigal [5]. Surprisingly, when infection was normalized in both infection systems by total viral DNA in target cells, the GFP signal was higher in cell-free viral infection. In summary, cell-associated infection is a more efficient mechanism in the sense of transmitting higher amounts of DNA per cell. Conversely, for the same amount of DNA, cell-free virus infection is able to spread HIV infection in a higher number of cells. These results provide new insights into the so accepted high efficiency of infection of cell-to-cell transmission.

While HIV Gag staining does not allow discriminating between non-infectious and infectious transfer events during cell-to-cell transmission, the use of cell lines containing reporter constructs can induce a replication-independent transactivation by a paracrine mechanism or through cell-cell fusion. Thus, we propose that the simplest manner to overcome discrepant results concerning the evaluation of antiretrovirals in both infection modes is to use assays that measure outcomes directly relevant to infection in target cell. These include the use of qPCR to measure *de novo* reverse transcribed viral DNA [53], single-cycle replication-dependent reporter vectors [149] or detection of *de novo* synthetized Gag production in target cells [18].

In conclusion, contacts formed between HIV-infected and uninfected target cells leads to the passive transfer of virus in the absence of viral replication and thus insensitive to the drug treatment. Consequently, markers commonly used to evaluate cell-free virus infection are not suitable when evaluating cell-to-cell virus transmission since they may

not correctly measure viral infectivity or drug efficacy. Moreover, active drugs completely block total DNA in cell-to-cell transmission, recapitulating the efficacy of antiretrovirals in cell free virus infections and *in vivo*. Understanding the causes that allow viral persistence in the face of ART may have important consequences for the development of new strategies to completely eradicate HIV. However, cell-to-cell transmission may not represent a mechanism able to generate ongoing replication during antiretroviral therapy.

Even though multiple studies have showed that memory CD4+ T cells support higher levels of HIV-1 replication than naïve CD4+ T cells [72-75], the mechanism underlying the different susceptibility to HIV-1 infection remains unclear. Since, memory and naïve T cells differ in their migratory capacity which may be mainly driven by cortical actin polymerization and cortical actin dynamics are required for receptor recruitment and viral cell-to-cell transfer, we speculated that differences in the cortical actin between naïve and memory T cell subsets may determine different susceptibilities to cell-to-cell transmission of HIV-1. We found that the degree of actin polymerization of target cells modulated the efficiency of viral antigen transfer. We also showed phenotypic differences in the cortical actin of naïve and memory T cells, exhibiting memory T cells higher cortical actin density than naïve T cells. Accordingly, the higher cortical actin density make memory CD4+ T cells more prone to capture viral antigens and consequently more susceptible to HIV-1 infection.

Consistent with previous reports showing that differences in susceptibility to HIV-1 infection between naïve and memory T cells were established during the initial stages of infection [76-78], we have shown that cortical actin regulates HIV-1 infection through modulating the uptake of viral antigens during cell-to-cell transfer. However, the main difference between these results and ours is the infection system used. Unlike cell-free virus infection, our short-term coculture model between HIV-infected cells and non-stimulated primary CD4+ T cells may maximise the endocytic component in target cells and hence its contribution in the final infection. Moreover, mechanisms that may actively contribute to cell-free virus transmission such as LFA-1/ICAM-1 interactions [78], may not be required during cell-to-cell transmission [39]. Additionally, the higher efficiency of cell-to-cell HIV transmission compared to cell-free virus infection [3, 18, 53] may allow to overcome restrictions observed during cell-free virus infection and thus may minimise the qualitative differences between both T cell subtypes which it

may explain that, unlike cell free virus infection [76], we did not see significant differences in viral fusion in both T cell subsets.

In contrast to our and other's results [72-75], one previous study found that X4 HIV-1 was preferentially transmitted to naïve T cells. Furthermore, the susceptibility to DC-mediated transmission into different CD4+ T cell subsets was attributed to the level of coreceptor expression [150]. Several possibilities may explain this discrepancy. First, cells in that report [150] were positively selected through fluorescence-activated cell sorting. Thus, all cell populations had antibodies attached to the surface during infection which probably affected viral entry. In contrast, our T cell subsets were identified after infection and had no antibodies attached. Second, the enhanced replication of X4 HIV-1 in naïve T cells following DC-mediated transmission may reflect differences between the DC-induced stimulation and other stimulation methods. In fact, memory T cells were more susceptible to X4 HIV-1 infection than naïve T cells upon CD3/CD28 stimulation [150]. However, consistent with other results [76, 77], we could not explain differences in the susceptibility to infection of naïve and memory T cell subsets by the different expression of viral coreceptors.

Only genuine HIV envelope could mediate infection of resting T cells while VSV-mediated endocytic entry which circumvents viral receptors and cortical actin was defective in non-dividing T cells [55], suggesting different requirements between both entry pathways but also unavoidable viral interactions with the actin cytoskeleton when infecting resting cells. Furthermore, recent findings have highlighted the contribution of the cortical actin during essential processes for the establishment of viral infection such as viral entry [124-126], reverse transcription [77] [98] or budding [37], presenting itself as a barrier but also as a factor required for infection. Specifically, the cortical actin may play a particularly relevant role during cell-to-cell transmission due to the characteristics of this transmission method requiring polarized budding of HIV particles in effector cells [37] or HIV-receptor clustering in target cells [30], processes in which the cortical actin may be directly involved. Proof of this is the different sensitivity of cell-free and cell-cell HIV-1 infections to the actin inhibitor cytochalasin D [32]. In summary, the infection system, the cell type, but also the degree of activation may impose different cortical actin requirements and may also condition the route of infection.

Thus, the distinct degree of actin polymerization in naïve and memory CD4+ T cells may induce different cellular receptor recruitment at the cell-cell contact zone affecting the efficiency of HIV-antigen internalization. On the other hand, differences in the actin polymerization may cause different rates of CXCR4 endocytosis and consequently different efficiencies of HIV-antigen uptake [136]. Either way, the higher viral antigen capture and the broader pattern of migration of memory CD4+ T cells [69] may contribute to disseminate infection more efficiently than naïve CD4+ T cells. Furthermore, cortical actin changes promoted during chemokine-directed recirculation [137] may favour infection of these HIV-loaded CD4+ T cells.

Determine the restriction imposed by the cortical actin in different T cell subsets and how the virus takes advantage of this machinery to infect is important for basic understanding of the HIV life cycle and has implications for viral pathogenesis. Although pharmacological treatment of the cytoskeleton may not be considered as a good therapeutic intervention due to the high toxicity of the actin inhibitors, modulating the cortical actin activity through interfering with downstream signalling molecules would be a more feasible option. In conclusion, the cortical actin determines differences in the susceptibility to HIV infection in naïve and memory CD4+ T cells by modulating the degree of HIV-antigen internalization.

Cell-to-cell transmission of HIV-1 is a highly efficient mechanism of virus infection as demonstrated with *in vitro* experiments and it may play a relevant role during local and systemic *in vivo* dissemination [24]. Actually, to shed light on the contribution of this mechanism during *in vivo* infection represents one of the incoming future challenges. Studies using lymphatic tissue samples were this mechanism probably has its maximum impact combined with new technological improvements would eventually clarify it.

Regarding the entry pathway, the latest evidences have reinforced the notion that endocytosis precedes virus entry into the cell cytoplasm of target cells. However, our results call for caution on the endocytic pathway as a productive entry route. Resolving questions regarding the kinetics of viral maturation into a fusion-competent state as well as specific HIV-receptor requirements may clarify discrepancies regarding the outcome of viral antigen transfer and what would be the productive infectious route.

Conceptually, spreading directly from infected to non-infected cells appears to have biophysic, kinetic and immunologic advantages compared to cell free-virus infection. However, our results call for optimism because the virus is blocked with equal efficacy in cell-free or cell-associated infections systems. Nonetheless, the efficacy of antiretrovirals could be compromised in anatomical sanctuary sites were drug concentrations may be lower. Thus, pharmacokinetic studies and new delivery strategies that improve the penetration of antiretrovirals are also a major challenge for the future of HIV field.

In summary, the basic research on the mechanism of cell-to-cell transmission of HIV-1 is important for understanding viral spread and pathogenesis and thus, it must be considered for future antiretroviral treatments and when developing of new strategies to cure HIV.

CONCLUSIONS

1. The internalization of HIV-1 antigens into intracellular compartments during cell-to-cell transfer requires the early endocytic machinery but is independent of endosomal maturation.

2. Endocytosis is not a mechanism of infection of primary CD4+ T cells, but may serve as a reservoir capable of inducing trans-infection of cells after the release of HIV-1 particles to the extracellular environment.

3. Intracellular detection of HIV-1 antigens in target cells after cell-to-cell transfer measures passive transfer of virus in the absence of viral replication and therefore, does not accurately measure infectivity or drug efficacy.

4. Cell-free and cell-associated HIV-1 infections are equally sensitive to inhibition by antiretroviral treatment. Thus, cell-to-cell HIV-1 transmission may not represent an escape mechanism able to generate residual replication during antiretroviral therapy.

5. The cortical actin density determines the susceptibility to cell-to-cell transmission of HIV-1 by modulating the degree of viral antigen internalization providing one explanation to the differences in the susceptibility to HIV-1 infection found in naïve and memory CD4+ T cells.

PUBLISHED ARTICLES IN PDF FORMAT

Review

Endocytosis of HIV: anything goes

Marc Permanyer, Ester Ballana and José A. Esté

Retrovirology Laboratory IrsiCaixa, Hospital Universitari Germans Trias i Pujol, Universitat Autónoma de Barcelona, 08916 Barcelona, Spain

The major pathway for HIV internalization in CD4+ T cells has been thought to be the direct fusion of virus and cell membranes, because the cell surface is the point of entry of infectious particles. However, the exact contribution of endocytic pathways to the infection of CD4+ T lymphocytes is unknown, and the mechanisms involved in endocytosis of HIV particles are unclear. Recent evidence suggests that endocytosis of cell-free and cell-associated virus particles could lead to effective virus entry and productive infections. Such observations have, in turn, spurred a debate on the relevance of endosomal entry as a mechanism of escape from the immune system and HIV entry inhibitors. In this paper, we review the endocytosis of HIV and discuss its role in HIV infection and pathogenesis.

Entry of HIV into CD4+ T cells

HIV, the causative agent of AIDS, is the most intensely studied infectious agent of all time. Research on the pathogenesis of HIV has led to an unprecedented level of control and management of AIDS, to the point that HIV infection has gone from being an inherently untreatable infectious agent to being considered a chronic treatable infection [1,2].

Soon after the discovery of HIV, viral entry into CD4+ T cells was recognized as an important step in the replication cycle of HIV and a possible target for antiviral intervention [3,4]. HIV envelope glycoproteins (gp120 and gp41) are the major inducers of neutralizing antibody responses, and these govern the viral entry process. In general, HIV enters target cells by a three-step process similar to that observed in other enveloped viruses. The first step involves the binding of a viral surface protein to receptors on the plasma membrane of the host cell. Although the CD4 molecule is the principal receptor in primary CD4+ T cells, other interactions beyond CD4–gp120 can be required for HIV-1 attachment to other cell types [5]. After virus attachment to the cell surface, HIV engages with the CD4 receptor, allowing additional interactions with a coreceptor protein (Figure 1). The major HIV-1 coreceptors are the chemokine receptors CCR5 and CXCR4. Expression of CCR5 or CXCR4 on different CD4+ target cells determines the permissiveness of the cells to infection by the corresponding CCR5-using (R5) or CXCR4-using (X4) HIV-1 strains. After virus attachment to CD4 and coreceptor engagement, theoretical models indicate that conformational changes in gp120 allow gp41 to reorient parallel to the viral and cellular membranes, and promote the events leading to virus and cell membrane fusion. Thereafter, the current working model to explain membrane fusion assumes the formation of a transient intermediate in which gp41 spans through both viral and cell membranes. This intermediate constitutes a target for gp41 derived inhibitory peptides. It is believed that a six-helix bundle gp41 structure forms before fusion, and serves to bring the membranes into close apposition, allowing the formation of the fusion pore and subsequent virus internalization [4].

The various steps in viral entry are relevant targets for anti-HIV intervention, and a large number of agents have been tested. Two of these, the gp41 fusion inhibitor enfuvirtide (T20, Fuzeon™, Roche, UK) and the CCR5 antagonist maraviroc (Selzentry™, Pfizer, USA), are currently in use for the treatment of drug-experienced individuals infected with HIV [4].

The events that lead to HIV entry have long been considered to occur at the cell surface [6]. Electron microscopy (EM) was initially used to identify the point of HIV entry by revealing fusion of viral and cell membranes, an observation that was further supported by the general understanding that HIV entry is pH independent [6,7]. Additional evidence came from the observation that HeLa cell lines expressing wild type or mutant CD4 molecules that severely impaired their ability to undergo endocytosis were equally susceptible to infection [8]. Moreover, endocytic internalization and endosomal acidification (a hallmark of endocytic entry of other enveloped viruses) appear not to be required to activate HIV entry into the cytoplasm [6,9–11]. Nevertheless, a variety of cell types such as macrophages, endothelial and epithelial cells and also lymphoid cells were observed to be able to bind and internalize HIV particles into vesicular structures [12–18]. Endosomes containing HIV particles were documented as early as 1988 [19]; however, it was not until recently that development of novel imaging technologies and novel approaches to inhibit HIV entry pathways provided insights into the role of endocytic virus entry in HIV replication [7,20], indicating that endocytosis might act as a relevant entry route for HIV.

There is an ongoing debate as to whether HIV-1 fuses predominantly at the cell surface, or from within an endosomal compartment, in order to enter cells. The original paradigm that HIV-1 fuses predominantly at the cell surface has been at least partially overturned by evidence suggesting that endocytosis can often precede virus–cell membrane fusion. Endocytosis preceding viral entry into the cell cytoplasm might be particularly relevant to direct cell–cell spread of HIV-1, but its ultimate relevance to *in vivo* spread of the virus has not been demonstrated to date. In this review, we discuss the recent developments in the study of HIV entry and the different experimental

Corresponding author: Esté, J.A. (jaeste@irsicaixa.es)

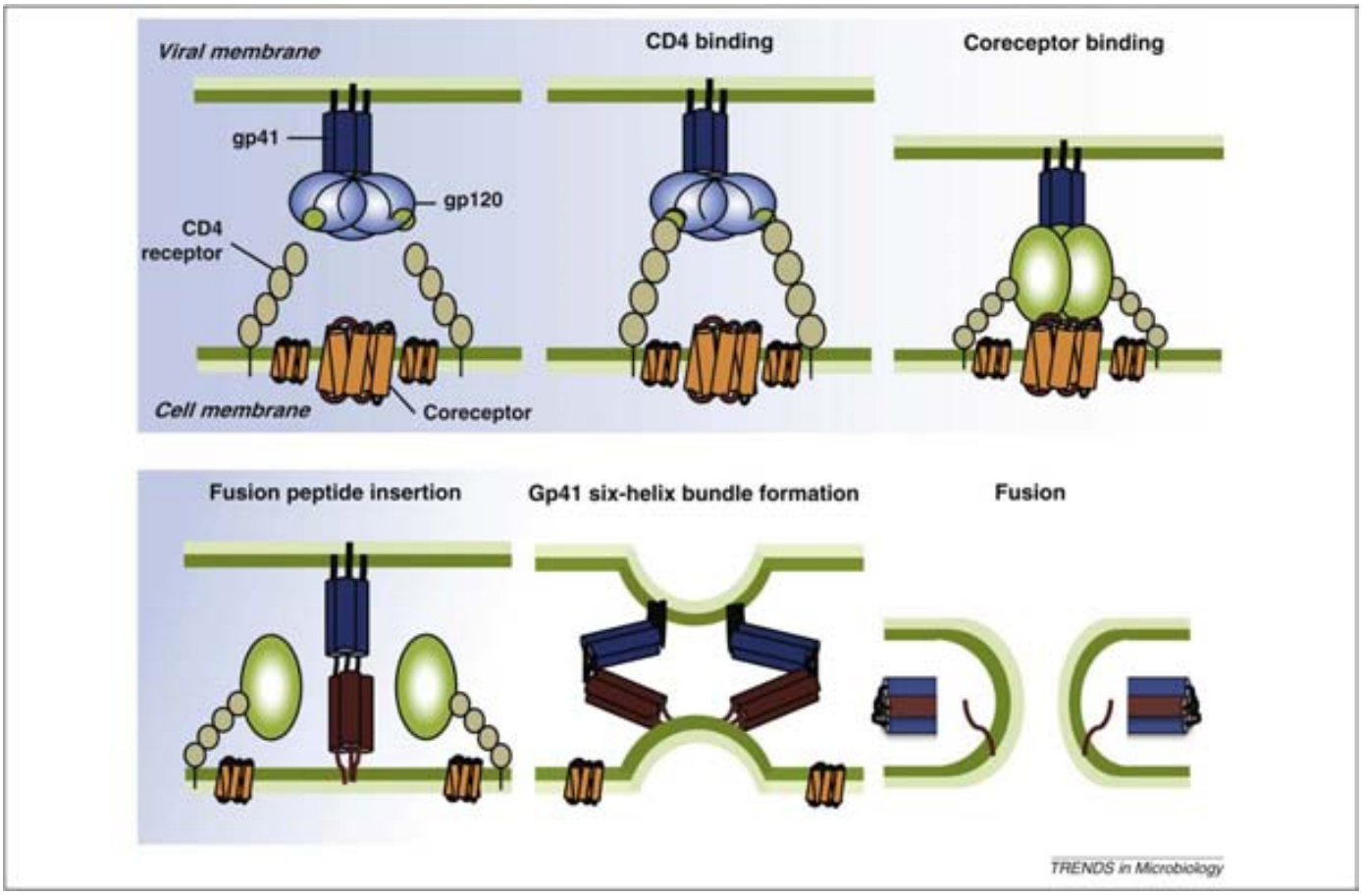


Figure 1. **The multistep process of HIV fusion.** The well-defined HIV fusion process leading to the delivery of the HIV viral core into the cytoplasm begins with the binding of the viral envelope glycoprotein gp120 with the cellular receptor CD4 (binding site for CD4 is shown in green). After gp120–CD4 engagement, conformational changes in gp120 trigger its interaction with the viral chemokine coreceptor CXCR4 or CCR5. Thereafter, gp41 is exposed into a fusogenic conformational state that triggers the insertion of the fusion peptide into the target cell. Finally, the six-helix bundle formation brings both membranes close together, leading to membrane fusion.

approaches that have caused the role of endocytic pathways in HIV infection to be revisited, with the aim of updating and formulating new perspectives in the field of HIV entry.

Virus endocytosis

The term 'endocytosis' includes at least four mechanisms: phagocytosis, macropinocytosis, clathrin mediated endocytosis, and caveolin mediated endocytosis [21,22], which differ in several properties such as vesicular size, markers and regulation. Different viruses use cellular endocytic mechanisms to enter and infect cells with the clathrin mediated pathway being the most commonly observed uptake pathway [22,23]). Once HIV is internalized, virus particles can follow different pathways: they can be secreted [24] (as in the case of transcytosis [12]) or degraded [25], or they can fuse with vesicular membranes to inject the viral core into the cytoplasm and initiate the viral infection cycle. However, the exact contribution of endocytic pathways to the infection of CD4+ T lymphocytes and to HIV pathogenesis *in vivo* is mostly unknown, and the mechanisms governing endocytosis also remain unclear.

Mechanism of HIV endocytosis: a clathrin mediated process

Fluorescent microscopy techniques have dramatically increased our ability to directly visualize and measure various stages of the HIV-1 life cycle [20], allowing the tracking of live HIV particles as they make their way into the cell, or the process by which viral proteins surf through the intracellular milieu, thereby allowing the confirmation of virus–host interactions by means of a 'see it to believe it' technical breakthrough. Recently, Hübner *et al*. [26] used confocal and transmission EM to describe the transfer of HIV particles between cells, a process that was preceded by the formation of the so-called virological synapse [27], cell–cell contacts involving cellular and viral proteins that promote and significantly increase the transfer of virus from infected to target cells. Live recording of cellular contacts between HIV-Gag antigen-containing cells and phytohemagglutinin (PHA)/interleukin (IL)-2-stimulated T cells were used to show how target cells are rapidly infected through concentrated buttons of HIV-Gag in polarized crescents contacting uninfected cells. This technique allow us to see what had already been demonstrated experimentally: intracellular HIV-Gag moves towards the cell surface to polarized regions that coincide with formation of the virological sypnase [27,28], penetration ofthe target cell, migration distantly from the cell surface [20,29], and dissociation from envelope glycoproteins if appropriate receptors and coreceptors are available, or in the absence of coreceptor, colocalizion with gp120 when internalized as complete virus particles [18,30]. Coreceptor independent internalization of HIV

occurs through an endocytic mechanism, leading to the detection of relatively large vesicular structures that contain apparently intact virus particles [18,30–32].

Hübner *et al.* [26] did not confirm whether HIV particles that were transferred to uninfected cells and contained in intracellular vesicles finally reached the cytoplasm, whether virus particles were able to uncoat, or whether an endocytosed virus was able to induce a productive infection. In addition, the work did not distinguish between the traditional virus–cell fusion entry and the newly proposed endocytic entry as the mechanism leading to productive infection in cell–cell transmission. Following this line of evidence, single particle imaging and a virus population based fusion assay were used by Miyauchi *et al.* [7] to evaluate the fate of HIV internalized through endosomes in TZM-bl cells (a modified CD4+, CCR5+, CXCR4+ HeLa cell line that allows detection and quantification of HIV capsid associated β-lactamase in the cytosol of infected cells). In this work, HIV particles were dually tracked by labeling viral envelope lipids and viral content with two independent fluorescent markers. The authors found that endocytosis of HIV particles could lead to internalization of complete virions that released their envelope at the cell perinuclear area before generating a β-lactamase positive signal, a marker of a productive infection. By contrast, virus particles that underwent fusion with the plasma membrane did not lead to productive infection.

Earlier, Daecke *et al.* [32] demonstrated the role of endocytosis in productive entry of HIV-1 to HeLa cells by using *trans* dominant negative proteins that interfere with specific clathrin endocytic routes. These proteins effectively block viral replication, whereas a caveolin-1 mutant that inhibits caveola dependent endocytosis had no significant effect on viral replication, clearly pointing to clathrin mediated endocytosis as a significant contributor to productive HIV entry. In addition, the size and ultrastructural evaluation of the vesicles formed in primary CD4+ T lymphocytes cocultured with persistently infected lymphoid cells or acutely infected primary lymphocytes pointed to a clathrin independent endocytic mechanism [18]. However, the observation that vesicles containing clathrin and dynamin also harbor the early endosomal marker early endosome antigen (EEA)1 but not caveolin-1 were suggestive of a clathrin mediated process [33] (Figure 2), contrasting with the results from Jolly *et al.*, [27] who did not observe colocalitzation between the Gag protein and EEA-1.

Miyauchi *et al.* [7] provided additional evidence by pretreating TZM-bl cells with dynasore, a small molecule inhibitor of the dynamin GTPase activity that prevents the scission of clathrin coated pits from the plasma membrane [34]. Dynasore pretreatment abolished the release of viral content but permitted lipid transfer to the plasma membrane, therefore preventing virus internalization and the subsequent steps that could lead to virus replication. Thus, HIV-1 particles appeared to enter cells through a clathrin and dynamin mediated process, and importantly, this indicated clathrin dependent endocytic entry as the mechanism leading to productive infection [7,35].

Is endocytosis an effective mechanism of HIV entry?

The results showing endocytosis to be the mode of HIV entry are in apparent contrast to previous reports that concluded that there was no overlap between HIV-1 antigen and early endosomes, and found no evidence of virus particles within endosomal structures formed in target cells that were exposed to persistently infected HIV-1 cells

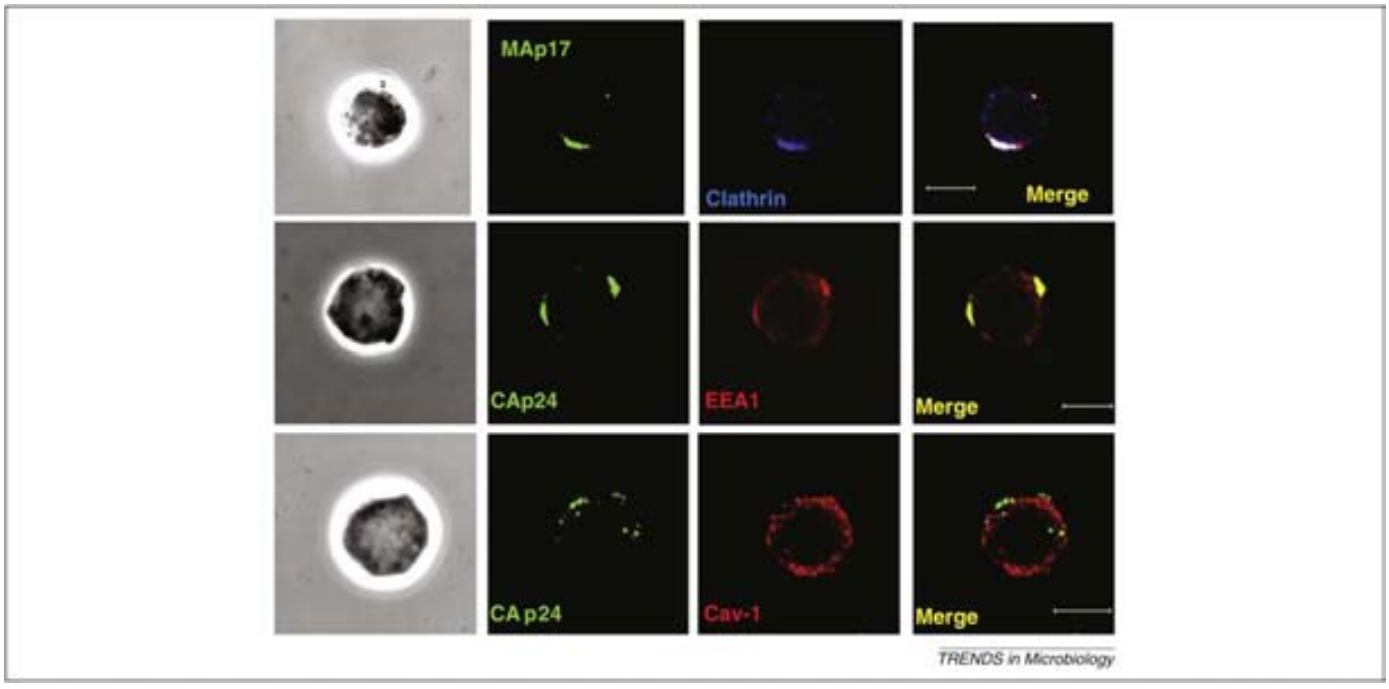


Figure 2. Transfer of HIV-1 particles into T CD4+ lymphocytes through a clathrin–dynamin dependent pathway. Primary T CD4+ lymphocytes were cocultured with MOLT-NL4-3 cells for 3 h. After coculturing, cells were stained for HIV antigen (matrix p17, MAp17 or capsid p24, Cap24) (MA, green), clathrin (blue) or caveolin (red). Colocalization is shown in white. Cells were identified by morphology. Scale bar = 5 μm. Reprinted with permission from Ref. [33].

[27,28,36]. Using EM or tomography, Martin *et al.* did not find endocytic uptake of HIV-1 by primary CD4+ T cells across virological synapses between HIV-1 BaL-infected Jurkat T cells and uninfected primary CD4+ T cells [36], suggesting that endocytosis of HIV particles might be rare in primary CD4+ T cells compared with the immortalized cell lines predominantly used in other analyses [7]. Indeed, some discrepancies in the mode of entry of HIV could be the result of differences in the variety of culture models and cell culture conditions (Table 1), including the ratio of infected to uninfected T cells, the chronicity of the infection in the effector cells, the type of cell or cell line used [36], and the degree of activation of target cells. Use of different experimental approaches could be the reason behind the great disparity of results from different laboratories. Table 1 lists some of the experimental settings used to characterize the mechanism of entry of HIV. For example, Chen *et al.* [37] reported absence of inhibition of virus transfer by

Table 1. The variability of the HIV route of entry: techniques, cells, viruses, and entry pathways

HIV entry pathway	Experimental model	Virus strain	Cell line	Strategies	HIV entry and infection marker	Refs
Direct fusion with the plasma membrane	Cell-free virus infection	HIV-1_{LAV-1}	Human T-lymphoblastic VB cell line	Lysosomotropic agents	Southern and slot blot analyses of low molecular weight DNA and electron microscopy	[6]
Direct fusion with the plasma membrane	Cell-free virus infection	HIV-1	HeLa cell line	Wild-type or mutant CD4 molecules	Multiple HIV infection assays including supernatant and cytoplasm viral antigen detection or syncitya formation	[8]
Receptor-mediated endocytosis	Cell-free virus infection	HIV-1_{LAV1-A}	Human T-lymphoid CEM cell line	OKT4A antibody pre-treatment	Kinetics of entry and uncoating of a radiolabeled virus and electron microscopy	[19]
Receptor-mediated endocytosis after inhibition of endosomal/lysosomal degradation	Cell-free virus infection	HIV-1_{SF2}	HeLa Magi indicator cells	Inhibitors of endosomal acidification	β-galactosidase expression with the X-Gal substrate	[25]
Dynamin-dependent, clathrin-mediated endocytosis	Cell-free virus infection	HIV-1_{NL4-3}, HIV-1_{SF2}, HIV-$_{1MVP8161}$	HeLa cell line	Dominant-negative mutants of dynamin and Eps15	β-lactamase assay [57]	[32]
Probably direct fusion with the plasma membrane (non-endocytic route)	Cell-to-cell cocultures	HIV-1_{LAI}	T lymphocytic Jurkat cell line and primary CD4+ T lymphocytes	Viral and cellular protein staining	Colocalitzation between viral antigens and with cellular proteins using confocal microscopy	[27]
No endocytic uptake observed in primary CD4+ T cells after virological synapse-dependent HIV-1 transfer	Cell-to-cell versus cell-free virus infection	HIV-1_{IIIB}, HIV-1_{BAL}	Human T lymphoblastic Jurkat cell line, A301.R5, primary CD4+ T lymphocytes	HIV entry inhibitors, time-lapse imaging of long-lived virological synapses	Electron microscopy and tomography. Detection of *de novo pol* DNA viral transcripts	[36]
Release of infectious HIV-1 particles after coreceptor-independent transfer	Cell-to-cell cocultures	HIV-1_{NL4-3}, HIV-1_{BAL}	T lymphoblastic MOLT4/CCR5 cell line	HIV entry inhibitors, the actin inhibitor cytochalasin D	Electron microscopy, intracellular and supernatant CAp24 antigen staining, syncytium formation, β-galactosidase assay	[53]
Clathrin-dependent endocytosis	Cell-to-cell cocultures	HIV-1_{BAL}	Monocyte-derived dendritic cells (DCs) and primary CD4+ T cells	HIV entry inhibitors	Intracellular and supernatant CAp24 antigen detection	[46]
Virological synapse-mediated HIV-1 transfer probably through an endocytic process	Cell-to-cell versus cell-free virus infection	HIV-1_{NL4-3} Gag-iGFP	Human T lymphoblastic Jurkat cell line, primary CD4+ T lymphocytes, MT4 cells	HIV entry inhibitors, actin inhibitor Cytochalasin D and viral mutants	Confocal microscopy, flow cytometric analysis	[37]
Virological synapse-mediated endocytosis	Cell-to-cell cocultures	HIV Gag-iGFP, HIV NL-GI, HIV which have GFP replacing the *nef* gene	Human T lymphoblastic Jurkat cell line, primary CD4+ T lymphocytes, MT4 cells	Gag-iGFP movements and GFP diffusion	Confocal and transmission electron microscopy	[26]
Dynamin-dependent endocytosis	Cell-free virus infection	HIV-1_{JRFL}, HIV-1_{HXB2}	HeLa-derived TZM-bl cells and the lymphoid CEM-SS cell line	Fusion inhibitors (i.e. C52L, a gp41-derived peptide), temperature, viral markers, the dynamin inhibitor dynasore	Confocal microscopy and β-lactamase assay	[52]

the CXCR4 coreceptor antagonist AMD3100, but also reported inhibition by the same agent in a later study [25] when a different surrogate marker of viral infection was measured (transcription of HIV-1 long terminal repeat driven green fluorescent protein [26,36] instead of confocal microscopy and flow cytometry based analyses [37]), highlighting the disparity of the results obtained.

An important point to take into account is the cell type used, that is, laboratory adapted cell lines or different types of primary cells (CD4+ T cells, dendritic cells, macrophages). Even though the major determinant for HIV-1 infection *in vivo* appears to be the CD4–coreceptor complex, virus binding to the cell surface, the first step in viral infection, can be regulated by several attachment factors, which in turn, can influence the infectivity of the target cell. In CD4+ T lymphocytes, the activated form of the integrin α4-β7 mediates the binding of gp120, which in turn activates the cell–cell spread machinery [38]. Interestingly, HIV-1 can bind to heparin sulfate proteoglycans (HSPGs), which appear to act as alternative attachment factors in HeLa cell lines before CD4 engagement [39]. In addition, several adhesion molecules can enhance viral attachment in a cell type dependent manner, which eventually can increase infectivity by up to an order of magnitude [5].

Other interactions besides the CD4–gp120 are required for HIV attachment to cells that express little CD4, such as macrophages, microglia and dendritic cells. Sindecans and C-type lectin receptors have been identified as attachment factors for macrophages and dendritic cells [40–43].

Some groups have used primary CD4+ T cells [18,30,33], whereas others have used lymphoid cell lines [36]; the difference between these might lie in the degree of activation of primary cells. Use of non-stimulated CD4+ primary T cells as targets implies that they are less prone to productive virus replication, express lower CXCR4 levels and thus are a better model for the evaluation of virus transmission in the absence of *de novo* virus production. Conversely, PHA/IL-2 activated cells express higher levels of coreceptors, and might easily fuse with infected cells and rapidly produce virus particles, impeding the identification of incoming viral antigens. Both activated T cells and lymphoid cell lines, commonly used for the evaluation of virus replication and HIV entry, might undergo virus–cell fusion rapidly, therefore masking endocytic virus transfer. Importantly, Yu *et al*. [44] have shown that in transformed T cell lines, pseudotyping HIV with the vesicular stomatitis virus envelope G protein (VSV-G) that induces viral entry through an endocytic process was able to induce higher nuclear migration of retrotranscribed viral DNA. Conversely, in resting CD4+ T cells, only the HIV envelope mediated process led to virus entry, DNA synthesis and nuclear migration, suggesting alternative routes of viral genome delivery by endocytic and non-endocytic processes, but also highlighting differences in the mechanism of viral entry in transformed or metabolically active cells compared with resting T cells [44]. Taking all the observations together, it is clear that evaluation of HIV transmission is strongly dependent on the target cell type employed. Laboratory adapted cell lines are a good model to study HIV replication as they represent an activated phenotype that enhances productive infection; however, they have low ability to accumulate HIV particles (HIV transfer) and might not completely mimic primary cells [45].

When considering a putative *in vivo* scenario, one of the key players in cell–cell HIV transmission are dendritic cells (DCs), which are antigen presenting cells. DCs take up HIV and then migrate to lymph nodes, where the virus is transferred to helper T-cells. DCs might transfer virus particles attached to their surfaces, a process designated 'trans-infection' [42,which does not require productive infection of the DC itself. Alternatively, attached virus could enter the DC and initiate a productive infection. *In vitro* cocultures of HIV-1 infected DCs with primary CD4+ T cells induced virus transmission to target cells that could not be reversed by treatment with trypsin, suggesting that the virus was retained in an intracellular compartment. EM revealed endosomes containing HIV particles in CD4+ T cells. Importantly, upon PHA/IL-2 stimulation of the trypsin treated purified CD4+ T cells, productive infection was observed, as measured by intracellular HIV-1 antigen staining and detection of infectious viruses in the cellular supernatant [46]. We believe that the infecting virus could not come from any other source but the endosomes. These results do not indicate that virus fused with the endosomal membranes initiates a productive infection; however, they further support evidence of endocytosis as an active mechanism of virus transfer that could lead to infection of primary CD4+ T cells under certain conditions.

HIV entry inhibitors and inhibition of HIV endocytosis

Several studies indicate that the process of endocytic HIV entry could be modified by pharmacological agents. Transient transfection or inducible expression of dominant negative mutants of dynamin or epidermal growth factor receptor substrate (EPS)15, a specific marker of clathrin dependent endocytosis, in a CD4+ HeLa cell line were able to significantly reduce HIV infection [32]. Dynasore blocked HIV-1 fusion and infection of TZM-bl cells [7]. These results suggest that targeting the endocytic machinery and the cascade of events leading to viral endocytosis could prevent HIV infection, provided that inhibition of the endocytic pathway is not detrimental to cell survival.

However, the effect of HIV entry inhibitors in endocytic entry is a rather controversial issue. Endocytosis of HIV appears to require the interaction of HIV gp120 with CD4 on the cell surface. Therefore, agents that block viral attachment to CD4+ T cells (such as the anti-CD4 monoclonal antibody Leu3a [18] and the recombinant CD4-based protein (CD4-IgG2, Pro152) [30]), prevent the endocytic cell–cell virus transfer of viruses to uninfected cells. Conversely, anti-HIV agents affecting a later step in the gp120–CD4 interaction (such as BMS-806 [47,48], coreceptor antagonists, gp41 dependent fusion peptides, neutralizing antibodies [49] targeting gp41 or reverse transcriptase inhibitors) do not block the transfer of HIV particles to intracellular compartments in target CD4+ T cells. For example, the fusion inhibitor enfuvirtide had no measurable effect on virological synapse mediated transfer, suggesting that uptake of virus into target cells does not require triggering of viral membrane fusion [37]. Interestingly, in cocultures of infected T cells or DCs with

CD4+ T cells, blockade of CXCR4 by AMD3100 induced an increase in endocytic transfer of X4 HIV [18,30].

The lack of inhibition of viral replication by entry inhibitors and neutralizing antibodies, together with previous observations that endocytosed virus did not reach the cytoplasm and could be lysed in acidified vesicles [6,8], suggested that HIV endocytosis is a dead end pathway. However, a number of internalized HIV-1 particles do not colocalize with Lamp1, a well known marker for the lysosomal degradative pathway [33], and trypsinized cells loaded with HIV particles appeared to discharge fully infective virus particles that, in the absence of entry inhibitors, were able to re-infect bystander CD4+ cells [18,30,33,50,5]1. Thus, endocytosed virus particles remained infectious, and were able to endure until an appropriate environment allowed productive infection. Moreover, based on their results with their cell culture system, Miyauchi *et al*. [52] postulated that HIV-1 entry occurs through sequential CD4 and coreceptor binding, followed by virus endocytosis. Therefore, HIV might become resistant to entry inhibitors that are membrane impermeable, including BMS806, the CXCR4 antagonist AMD3100, and the gp41 derived peptide C52L, if added after virus particles have been endocytosed [7]. Altogether, these results define endocytosis as an itinerant virus reservoir that offers protection from the immune system but also from possible pharmacological inhibitors of HIV entry.

A working hypothesis: the kinetics of virus fusion as a determinant of the mechanism of HIV entry

Despite the large body of evidence discussed above supporting the possibility of a productive HIV infection set by an endocytosed virus, alternative mechanisms should not be underestimated. Three non-exclusive alternative routes of HIV entry can be considered, all of which might cause a productive HIV infection (Figure 3). In the first potential route, the cell surface could be the point of entry, a mechanism that has historically been considered the canonical mode of HIV entry. The second potential route could use a mechanism by which CD4 binding and coreceptor engagement leads to endocytic internalization, and virus–cell fusion occurs with the endosomal membrane [7,26,32]. The third pathway could be a coreceptor independent transfer of virus particles that could then lead to recycling or lysis of infectious virions [18,33,46]. We envision that all three alternatives are variants of the same mechanism and, by extension, all three probably coexist [31] in a delicate balance, which could tilt to one of the three alternatives depending on multiple cellular and viral factors affecting the kinetics of virus fusion. The variety of results obtained under different experimental conditions might reflect the *in vivo* variability and might be only a simplification of the true situation. In fact, the rate of the HIV gp120 virus engagement with cell surface CD4 and its appropriate coreceptor could hypothetically explain how this balance tilts to a particular route of entry. In addition, the rate of CD4 and

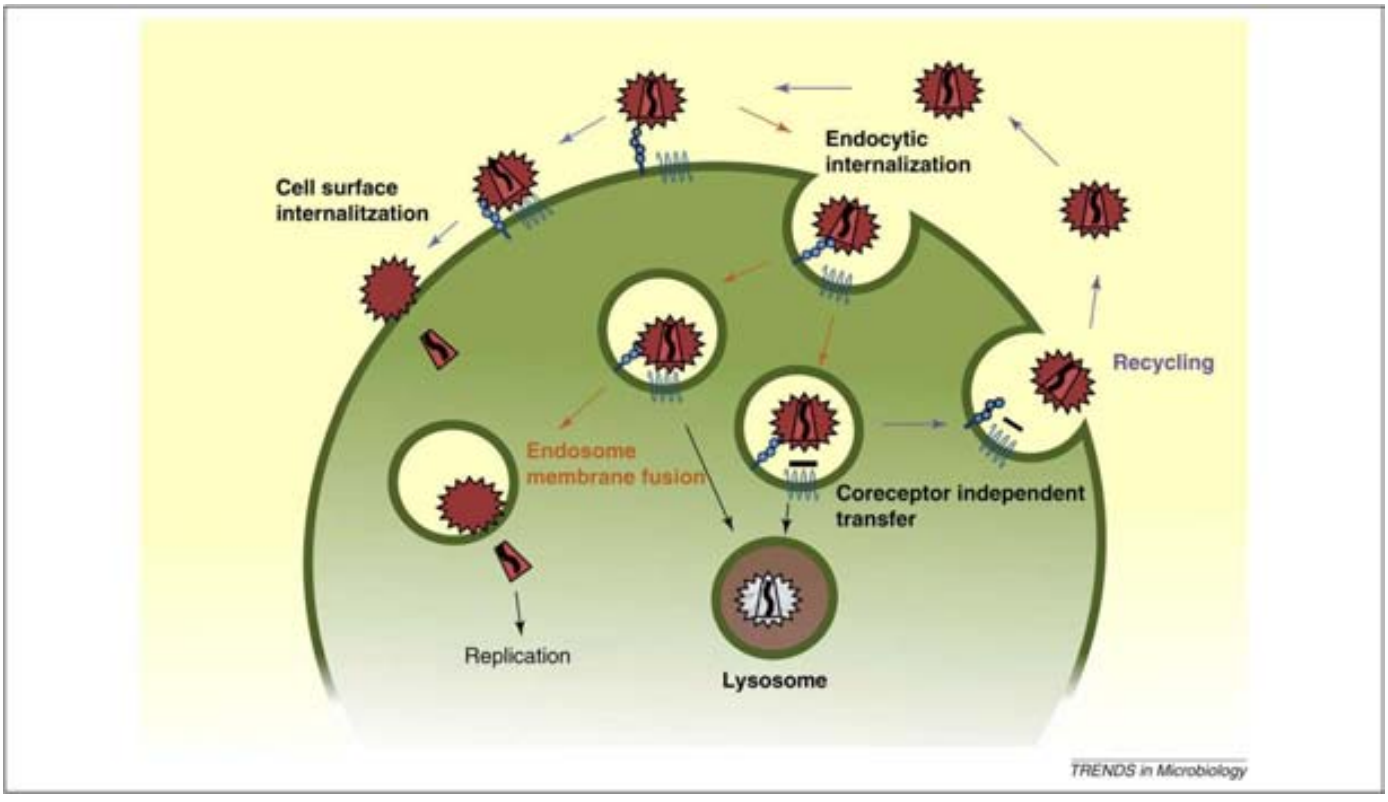


Figure 3. Putative HIV entry pathways. After binding of HIV to the CD4 receptor, the virus can enter target cells through three non-exclusive pathways. The first pathway is the canonical or direct fusion of HIV particles with the plasma membrane (cell surface internalization). In the second pathway, endosomal internalization establishes HIV fusion with an endosome membrane following an endocytic uptake of virus particles, or this could lead to endosome maturation and lysis of the virus particle. In the third pathway, coreceptor engagement might lead to virus membrane and endosome membrane fusion and release of the virion into the cytosol; alternatively, in the absence of the appropriate coreceptor or coreceptor independent transfer, viruses might be recycled back to the extracellular medium as infectious particles capable of mediating a productive infection.

coreceptor binding by HIV might also define the sensitivity to HIV entry inhibitors. In turn, the multiplicity of infection (i.e. the number of infectious particles per target cell), the availability of CD4, the degree of coreceptor expression, the activation state of the target cell, and the lipid compositions of virus and cell membranes can also affect the kinetics of virus entry. Therefore, different HIV-1 strains could have different HIV-1 fusion rate constants [7].

We propose a working model in which the kinetics of viral fusion is the driving force towards the three alternative modes of virus entry. After cell-free or cell-associated virus particles reach the target cell surface and engage the CD4 receptor, rapid fusion kinetics at the cell membrane promoted by envelope glycoproteins with high affinity for receptors and a high degree of receptor or coreceptor expression and/or cell activation, might favor virus–cell membrane fusion, leading to a productive infection. In some cases, complete fusion could be aborted despite virus–cell lipid mixing (hemifusion) at the cell membrane, inducing envelope dependent mononuclear cell death [53,54]. Conversely, low CD4 or coreceptor expression and/or low cellular activation drive low fusion kinetics (exemplified by the presence of HIV entry inhibitors at the time of virus–cell contacts) [30], leading to the accumulation of virus particles at the cell surface and subsequently the engulfment of virus particles by the target cell [18,33], from which some virus particles will proceed to lysosomes, others will fuse with the endosome membrane, and some might be recycled back to the cell surface [33]. Upon T cell activation and in the absence of HIV entry inhibitors, the recycled virus might induce a productive infection when conditions of high fusion kinetics are present [46]. Then, in an intermediate condition, as illustrated by Melykian *et al.* [7,5]2, the relatively low rate of fusion causes the engagement of receptor and coreceptor, which is sufficiently low to allow the engulfment of HIV particles by endocytosis before fusion with the cell membrane. The receptor and coreceptor availability inside the endosome promotes virus cell fusion within the endosomal compartment, leading to virus entry into the cytosol.

Role of virus endocytosis in the pathogenesis of HIV

The efficiency of HIV-1 spread between T cells *in vitro* is approximately 10-fold greater than that of cell-free viral spread [36]. Although no direct evidence exists regarding HIV spread efficiency *in vivo*, the number of cell–cell contacts are high, and cells carrying HIV particles or infected with HIV might increase virus spread by several fold compared with cell-free virus. The observation that under certain conditions (such as the presence of HIV entry inhibitors or neutralizing antibodies), endocytic cell–cell virus transfer is enhanced, was interpreted as a mechanism of virus escape from antiviral agents [26,30,37]. If proven, endocytosis could provide HIV with a selective advantage that does not require genotype or phenotype changes to serve as a mechanism of escape from antiviral drugs. Endocytosed viruses could hide without leaving any evidence of their presence. Hijacking the endocytic machinery might also allow HIV to avoid processing and exposure of viral antigens to the immune system, thus delaying the onset of an antiviral response. Endocytosis might not be the primary mode of entry leading to virus replication, but it could serve as a reservoir that transiently protects the virus from a negative environment.

Massanella *et al.* showed that anti-gp41 neutralizing antibodies blocked productive infection of target cells [49]. However, HIV-1 entry inhibitors have been shown to interfere equally with cell–cell and cell-free HIV-1 infection when inhibitors are added at the time of mixing target cells with infected cells or cell-free virus, suggesting that cell–cell mediated spread of HIV-1 is unlikely to be an antibody or drug evasion strategy for the virus [36,49]. These results are in line with the potency and efficacy demonstrated by HIV entry inhibitors. However, these evaluations have not taken into account the presence of anti-HIV drugs during all times of the evaluation of the infectivity of endocytosed virus particles. Thus, cells containing endocytosed virus might act as carriers of HIV to tissues and organs, freeing their cargo at these locations and then either becoming infected themselves or allowing bystander CD4+ T cells to become infected.

Conclusions and future perspectives

Involvement of endocytic pathways in HIV entry adds further complexity to the mechanism of virus replication and the pathogenesis of HIV and AIDS. The intricate balance between cell surface and endocytosis mediated entry requires further research to resolve the importance of each component in virus replication (Box 1). Despite some evidence of endosome-containing HIV particles in lympoid tissue, the role of endocytic entry in the natural history of HIV infection and disease outcome has not been defined. In particular, how HIV is transmitted in tissues where cell–cell contacts greatly increase viral spread or in body compartments that cannot be reached by antiviral agents is still unclear. In this context, the delivery of endocytosed virus could play a relevant role. Additional video and ultrastructural studies should determine the level of receptor and coreceptor expression in virus-containing intracellular vesicles, the possibility of virus particles from endosomes in T cells fusing into the cytoplasm, and the role, if any, of the endocytic process in the replication cycle and pathogenesis of HIV.

Box 1. Outstanding questions

1. What is the true contribution of endocytic virus entry to the infection and pathogenesis of HIV?
2. What are the structural and mechanistic requirements for virus–cell interactions leading to endocytosis of HIV? Elucidation will be required to decipher the role of endocytosis in virus entry.
   - What cellular factors play a role in endocytic HIV entry?
   - What are the viral determinants of endocytic virus entry?
   - Do virus strains differ in their capacity to promote endocytosis?
   - Is the rate of virus–cell membrane fusion a determinant of virus endocytosis?
3. What are the experimental settings that better model virus internalization *in vivo*?
4. Is virus endocytosis a reservoir or a mechanism of escape to HIV entry inhibitors, neutralizing antibodies and antiretroviral therapy?
5. Are cellular cofactors involved in endocytic virus entry potential targets for anti-HIV intervention?

Furthermore, the molecular mechanisms governing the endocytosis of HIV have not been clearly characterized. Several cellular cofactors affect virus replication and disease, which in turn, could be the target for antiviral intervention [55,56]. A large number of cellular cofactors are involved in clathrin mediated endocytosis or in other endocytic processes [22,57], some of which could be essential for HIV entry. Cell surface proteins such as integrins have emerged as cofactors and receptors for a large number of animal and human viruses that enter the cells through an endocytic process. Integrins, often involved in the entry of other animal and human viruses, appear to stabilize the virological synapse, serve as cofactors for HIV entry, and promote HIV-1 infection [38,58,59], suggesting that HIV, similar to other viruses, could also employ integrins for its own benefit. Elucidating the cascade of events leading to HIV endocytosis will provide conclusive evidence of this entry mechanism as a potential antiviral strategy.

Acknowledgements

Work in our laboratory was funded by the Spanish *Ministerio de Ciencia e Innovación* projects BFI2003-00405, BFU2006-00966, FIS PI060624, BFU2009-06958, FIPSE 360783-09 and the FP6 project LSHB-CT-03-503480. MP and EB are research fellows from Fondo de Investigación Sanitaria (FIS).

References

1 Esté, J.A. and Cihlar, T. (2010) Current challenges in antiretoviral research and treatment. *Antiviral Res.* 85, 26–33
2 Broder, S. (2010) The development of antiretroviral therapy and its impact on the HIV-1/AIDS pandemic. *Antiviral Res.* 85, 1–18
3 Este, J.A. and Telenti, A. (2007) HIV entry inhibitors. *Lancet* 370, 81–88
4 Tilton, J.C. and Doms, R.W. (2010) Entry inhibitors in the treatment of HIV-1 infection. *Antiviral Res.* 85, 91–100
5 Ugolini, S. *et al.* (1999) HIV-1 attachment: another look. *Trends Microbiol.* 7, 144–149
6 Stein, B.S. *et al.* (1987) pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. *Cell* 49, 659–668
7 Miyauchi, K. *et al.* (2009) HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. *Cell* 137, 433–444
8 Maddon, P.J. *et al.* (1988) HIV infection does not require endocytosis of its receptor, CD4. *Cell* 54, 865–874
9 Bedinger, P. *et al.* (1988) Internalization of the human immunodeficiency virus does not require the cytoplasmic domain of CD4. *Nature* 334, 162–165
10 Kielian, M. and Jungerwirth, S. (1990) Mechanisms of enveloped virus entry into cells. *Mol. Biol. Med.* 7, 17–31
11 Skehel, J.J. and Wiley, D.C. (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu. Rev. Biochem.* 69, 531–569
12 Bomsel, M. (1997) Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier. *Nat. Med.* 3, 42–47
13 Fackler, O.T. and Peterlin, B.M. (2000) Endocytic entry of HIV-1. *Curr. Biol.* 10, 1005–1008
14 Goto, T. *et al.* (1998) The life-cycle of human immunodeficiency virus type 1. *Micron* 29, 123–138
15 Liu, N.Q. *et al.* (2002) Human immunodeficiency virus type 1 enters brain microvascular endothelia by macropinocytosis dependent on lipid rafts and the mitogen- activated protein kinase signaling pathway. *J. Virol.* 76, 6689–6700
16 Marechal, V. *et al.* (2001) Human immunodeficiency virus type 1 entry into macrophages mediated by macropinocytosis. *J. Virol.* 75, 11166–11177
17 Schaeffer, E. *et al.* (2004) Compensatory link between fusion and endocytosis of human immunodeficiency virus type 1 in human CD4 T lymphocytes. *J. Virol.* 78, 1375–1383
18 Blanco, J. *et al.* (2004) High level of coreceptor-independent HIV transfer induced by contacts between primary CD4 T cells. *J. Biol. Chem.* 279, 51305–51314
19 Pauza, C.D. and Price, T.M. (1988) Human immunodeficiency virus infection of T cells and monocytes proceeds via receptor-mediated endocytosis. *J. Cell Biol.* 107, 959–968
20 Campbell, E.M. and Hope, T.J. (2008) Live cell imaging of the HIV-1 life cycle. *Trends Microbiol.* 16, 580–587
21 Conner, S.D. and Schmid, S.L. (2003) Regulated portals of entry into the cell. *Nature* 422, 37–44
22 Marsh, M. and Helenius, A. (2006) Virus entry: open sesame. *Cell* 124, 729–740
23 Mercer, J. *et al.* (2010) Virus entry by endocytosis. *Annu. Rev. Biochem.* 79, 803–833
24 Vidricaire, G. *et al.* (2003) The low viral production in trophoblastic cells is due to a high endocytic internalization of the human immunodefiency virus type 1 and can be overcome by the pro-inflammatory cytokines TNF-alpha and IL-1. *J. Biol. Chem.* 25, 25
25 Fredericksen, B.L. *et al.* (2002) Inhibition of endosomal/lysosomal degradation increases the infectivity of human immunodeficiency virus. *J. Virol.* 76, 11440–11446
26 Hubner, W. *et al.* (2009) Quantitative 3D video microscopy of HIV transfer across T cell virological synapses. *Science* 323, 1743–1747
27 Jolly, C. *et al.* (2004) HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse. *J. Exp. Med.* 199, 283–293
28 Martin, N. and Sattentau, Q. (2009) Cell-to-cell HIV-1 spread and its implications for immune evasion. *Curr. Opin. HIV AIDS* 4, 143–149
29 McDonald, D. *et al.* (2002) Visualization of the intracellular behavior of HIV in living cells. *J. Cell Biol.* 159, 441–452
30 Bosch, B. *et al.* (2005) Inhibition of coreceptor independent cell-to-cell HIV-1 transmission by a CD4-IgG2 fusion protein. *Antimicrob. Agents Chemother.* 49, 4296–4304
31 Ruggiero, E. *et al.* (2008) Virological consequences of early events following cell-cell contact between human immunodeficiency virus type 1-infected and uninfected CD4+ cells. *J. Virol.* 82, 7773–7789
32 Daecke, J. *et al.* (2005) Involvement of clathrin-mediated endocytosis in human immunodeficiency virus type 1 entry. *J. Virol.* 79, 1581–1594
33 Bosch, B. *et al.* (2008) A clathrin-dynamin-dependent endocytic pathway for the uptake of HIV-1 by direct T cell-T cell transmission. *Antiviral Res.* 80, 185–193
34 Macia, E. *et al.* (2006) Dynasore, a cell-permeable inhibitor of dynamin. *Dev. Cell* 10, 839–850
35 Uchil, P.D. and Mothes, W. (2009) HIV entry revisited. *Cell* 137, 402–404
36 Martin, N. *et al.* (2010) Virological synapse-mediated spread of human immunodeficiency virus type 1 between T cells is sensitive to entry inhibition. *J. Virol.* 84, 3516–3527
37 Chen, P. *et al.* (2007) Predominant mode of human immunodeficiency virus transfer between T cells is mediated by sustained Env-dependent neutralization-resistant virological synapses. *J. Virol.* 81, 12582–12595
38 Arthos, J. *et al.* (2008) HIV-1 envelope protein binds to and signals through integrin [alpha]4[beta]7, the gut mucosal homing receptor for peripheral T cells. *Nat. Immunol.* 9, 301–309
39 Mondor, I. *et al.* (1998) Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans. *J. Virol.* 72, 3623–3634
40 Saphire, A.C. *et al.* (2001) Syndecans serve as attachment receptors for human immunodeficiency virus type 1 on macrophages. *J. Virol.* 75, 9187–9200
41 de Witte, L. *et al.* (2007) Syndecan-3 is a dendritic cell-specific attachment receptor for HIV-1. *Proc. Natl. Acad. Sci. U. S. A.* 104, 19464–19469
42 Geijtenbeek, T.B. *et al.* (2000) DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* 100, 587–597
43 Lambert, A.A. *et al.* (2008) The C-type lectin surface receptor DCIR acts as a new attachment factor for HIV-1 in dendritic cells and contributes to trans- and cis-infection pathways. *Blood* 112, 1299–1307
44 Yu, D. *et al.* (2009) The HIV envelope but not VSV glycoprotein is capable of mediating HIV latent infection of resting CD4 T cells. *PLoS Pathog.* 5, e1000633

45 Puigdomenech, I. *et al.* (2008) HIV transfer between CD4 T cells does not require LFA-1 binding to ICAM-1 and is governed by the interaction of HIV envelope glycoprotein with CD4. *Retrovirology* 5, 32
46 Clotet-Codina, I. *et al.* (2009) HIV endocytosis after dendritic cell to T cell viral transfer leads to productive virus infection. *Antiviral Res.* 83, 94–98
47 Si, Z. *et al.* (2004) Small-molecule inhibitors of HIV-1 entry block receptor-induced conformational changes in the viral envelope glycoproteins. *Proc. Natl. Acad. Sci. U. S. A.* 101, 5036–5041
48 Gonzalez-Ortega, E. *et al.* (2010) ADS-J1 inhibits HIV-1 entry by interacting with gp120 and does not block the fusion-active gp41 core formation. *Antimicrob. Agents Chemother.* 54, 4487–4492
49 Massanella, M. *et al.* (2009) Antigp41 antibodies fail to block early events of virological synapses but inhibit HIV spread between T cells. *AIDS* 23, 183–188
50 Blanco, J. *et al.* (2005) Multiparametric assay to screen and dissect the mode of action of anti-human immunodeficiency virus envelope drugs. *Antimicrob. Agents Chemother.* 49, 3926–3929
51 Bosch, B. *et al.* (2006) Inhibition of human immunodeficiency virus type 1 infection in macrophages by an alpha-v integrin blocking antibody. *Antiviral Res.* 69, 173–180
52 Miyauchi, K. *et al.* (2009) Early steps of HIV-1 fusion define the sensitivity to inhibitory peptides that block 6-helix bundle formation. *PLoS Pathog.* 5, e1000585
53 Blanco, J. *et al.* (2004) R5 HIV gp120-mediated cellular contacts induce the death of single CCR5-expressing CD4 T cells by a gp41-dependent mechanism. *J. Leukoc. Biol.* 76, 804–811
54 Barretina, J. *et al.* (2003) Anti-HIV-1 activity of enfuvirtide (T-20) by inhibition of bystander cell death. *Antivir Ther.* 8, 155–161
55 Greene, W.C. *et al.* (2008) Novel targets for HIV therapy. *Antiviral Res.* 80, 251–265
56 Adamson, C.S. and Freed, E.O. (2010) Novel approaches to inhibiting HIV-1 replication. *Antiviral Res.* 85, 119–141
57 Robinson, M.S. (2004) Adaptable adaptors for coated vesicles. *Trends Cell Biol.* 14, 167–174
58 Ballana, E. *et al.* (2009) Cell adhesion through alphaV-containing integrins is required for efficient HIV-1 infection in macrophages. *Blood* 113, 1278–1286
59 Cicala, C. *et al.* (2009) The integrin {alpha}4{beta}7 forms a complex with cell-surface CD4 and defines a T-cell subset that is highly susceptible to infection by HIV-1. *Proc. Natl. Acad. Sci. U. S. A.* 106, 20877–20882

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 38, pp. 32017–32026, September 14, 2012

Trans-infection but Not Infection from within Endosomal Compartments after Cell-to-cell HIV-1 Transfer to CD4$^+$ T Cells*

Received for publication, January 16, 2012, and in revised form, July 23, 2012 Published, JBC Papers in Press, July 30, 2012, DOI 10.1074/jbc.M112.343293

Marc Permanyer[1], **Ester Ballana**[1], **Roger Badia, Eduardo Pauls**[2], **Bonaventura Clotet, and José A. Esté**[3]

From the IrsiCaixa, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, 08916 Badalona, Spain

Background: Endocytosis has been postulated as a route of HIV entry into cells.
Results: Endocytosed virus led to productive infection of cells, except when cells were cultured with the anti-gp120 antibody IgGb12.
Conclusion: Endocytosis may serve as a virus reservoir capable of inducing trans-infection.
Significance: The route of HIV transmission is important for understanding pathogenesis and drug therapy.

Cellular contacts between HIV-1-infected donor cells and uninfected primary CD4$^+$ T lymphocytes lead to virus transfer into endosomes. Recent evidence suggests that HIV particles may fuse with endosomal membranes to initiate a productive infection. To explore the role of endocytosis in the entry and replication of HIV, we evaluated the infectivity of transferred HIV particles in a cell-to-cell culture model of virus transmission. Endocytosed virus led to productive infection of cells, except when cells were cultured in the presence of the anti-gp120 mAb IgGb12, an agent that blocks virus attachment to CD4, suggesting that endocytosed virus was recycled to the outer cell surface. Confocal microscopy confirmed the colocalization of internalized virus antigen and the endosomal marker dynamin. Additionally, virus transfer, fusion, or productive infection was not blocked by dynasore, dynamin-dependent endosome-scission inhibitor, at subtoxic concentrations, suggesting that the early capture of virus into intracellular compartments did not depend on endosomal maturation. Our results suggest that endocytosis is not a mechanism of infection of primary CD4 T cells, but may serve as a reservoir capable of inducing trans-infection of cells after the release of HIV particles to the extracellular environment.

Viruses are obligatory intracellular parasites that take advantage of the host cell machinery to replicate and spread from infected to uninfected cells (1–3). Cell-to-cell transmission has been shown to be a highly efficient mechanism of virus spread (4, 5), and its relevance for *in vivo* dissemination in the active sites of replication, namely, primary and secondary lymphoid tissues, seems probable. HIV may be transferred from infected to uninfected CD4$^+$ cells (6, 7) by a mechanism that requires intimate cell-to-cell contacts involving the HIV envelope glycoprotein gp120 and the CD4 receptor but also accessory cell surface proteins (8). Virus-cell fusion and initiation of a productive infection require engagement to CD4 and to one of the two alternative coreceptors, CCR5 or CXCR4. The various steps in the mechanism of virus entry are considered targets for anti-HIV intervention (9, 10).

Cell-to-cell transfer of HIV particles may be blocked by agents that prevent virus attachment, such as the anti-CD4 monoclonal antibody (mAb) Leu3a, the anti-gp120 mAb IgGb12, or the CD4-IgG2 fusion protein PRO542 (11), but is resistant to HIV entry inhibitors targeting virus coreceptors or gp41-dependent fusion (7, 12), suggesting that virus attachment to CD4 is the sole factor necessary to induce the uptake of HIV particles (13) and that virus capture may occur in the absence of virus fusion and the initiation of a productive infection. Endocytic internalization and endosomal acidification have been shown not to be required to activate HIV entry into the cytoplasm (14–17).

Alternatively, several lines of evidence support clathrin-dependent endocytosis as an infectious pathway (12, 18–21). HIV fusion with endosomal membranes has been observed by electron microscopy (22). Daecke *et al.* (23) proposed a role for endocytosis in productive entry of HIV-1 by using trans-dominant negative proteins that interfered with specific clathrin-endocytic routes and effectively blocked virus replication. Complete fusion of HIV particles with HeLa cells has been observed to occur within endosome membranes (20), but complete fusion was blocked when endocytosis was inhibited (24). Recent data suggest that after cell-to-cell transfer, virions first need to undergo maturation within endosomes, delaying membrane fusion and reducing sensitivity to patient antisera compared with cell-free virus (25). Thus, the role of endocytosis in HIV replication and whether or not endocytic virus transfer represents an escape mechanism from the immune system or therapeutic agents remain highly controversial (5, 26).

Here, we show that primary CD4$^+$ T lymphocytes take up virus particles into dynamin-containing compartments even in the presence of the endosome-scission inhibitor dynasore. Moreover, purified cells carrying endocytosed virus particles did not become productively infected if cultured in the pres-

* This work was supported in part by the Spanish Ministerio de Economía y Competitividad Projects BFU2009-06958 and SAF2010-21617-C02, FIPSE 360783-09, and Gala contra la SIDA 2011.
[1] Research fellows from the Fondo de Investigación Sanitaria.
[2] Research fellow from the Spanish Ministerio de Economía y Competitividad.
[3] To whom correspondence should be addressed: IrsiCaixa, Hospital Germans Trias i Pujol, Canyet s/n, 08916 Badalona, Spain. Fax: 34-934653968; E-mail: jaeste@irsicaixa.es; jaeste@yahoo.com.

ence of HIV attachment inhibitors such as the anti-gp120 mAb IgGb12, suggesting that endocytosed virus was recycled to the cell surface to initiate a productive virus infection.

EXPERIMENTAL PROCEDURES

Cells—Peripheral blood mononuclear cells from healthy donors were purified by Ficoll-Hypaque sedimentation. $CD4^+$ T lymphocytes were immediately purified (>95%) from peripheral blood mononuclear cells by negative selection using the $CD4^+$ T cell enrichment kit (Stem Cell Technologies, Vancouver, Canada) and grown in RPMI 1640 L-glutamine medium (Invitrogen) supplemented with 10% (R10) heat-inactivated fetal calf serum (FCS; Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin. When needed, $CD4^+$ T cells were stimulated with phytohemagglutinin (PHA; Sigma) at 4 μg/ml and 6 units/ml interleukin 2 (IL-2; Roche Applied Science). MOLT-4 lymphoid cells (AIDS Reagent Program, National Institutes of Health, Bethesda, MD) were cultured in R10. Chronically HIV-1-infected MOLT cells were generated after the infection of MOLT cells with the NL4-3 X4 HIV-1 ($MOLT_{NL4-3}$) (23, 24). After the infection peak, the persistently infected culture was grown and characterized for Env expression and virus production. HEK293-T cells (AIDS Reagent Program) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Cocultures of Infected and Uninfected Cells—Nonstimulated primary $CD4^+$ T cells (to minimize virus replication) were cocultured with uninfected or HIV-1 persistently infected $MOLT_{NL4-3}$ cells as previously described (11, 18, 19). Purified $CD4^+$ T cells were first labeled with the cell tracker CMFDA (Molecular Probes) and washed before being mixed with $MOLT_{NL4-3}$ cells. Briefly, 2.5×10^6 of both infected and target cells (1:1 ratio) were cocultured in 48-well culture plates in a final volume of 1 ml in the absence or presence of the following HIV-1 inhibitors: 80 nM neutralizing anti-gp120 mAb IgGb12 (Polymun Scientific, Wien, Austria); 4 μM reverse transcriptase (RT) inhibitor 3-azido-3-deoxythymidine (AZT)[4]; 12.5 μM AMD3100 or 80 μM dynamin inhibitor dynasore (all from Sigma-Aldrich). Cocultures were incubated overnight at 37 °C. The capture of CAp24 antigen by primary $CD4^+$ T cells was evaluated by flow cytometry as shown before (10, 11, 25, 27, 28). Prior to staining, cells were trypsinized to eliminate HIV-1 particles bound to the cell surface. For trypsin treatment, cells were washed with phosphate-buffered saline (PBS) and treated for 8 min at room temperature with 0.25% trypsin solution (Invitrogen). Trypsin was stopped by addition of FCS, and cells were then washed with PBS. For intracellular staining, cells were fixed, permeabilized (Fix & Perm; Caltag, Burlingame, CA), and stained with the anti-HIV-CAp24 antigen mAb KC57 (Coulter). Cells were analyzed in a LSRII flow cytometer (BD Bioscience) and identified by morphological parameters and CMFDA staining.

[4] The abbreviations used are: AZT, 3-azido-3-deoxythymidine; BFLA1, bafilomycin A1; CMFDA, 5-chloromethylfluorescein diacetate; CON A, concanamycin A; PHA, phytohemagglutinin; PMA, phorol 12-myristate 13-acetate.

Isolation of Target $CD4^+$ T Cells—CMFDA-loaded target $CD4^+$ T cells were purified (>99% purity) from $MOLT_{NL4-3}$ cells by fluorescence-activated cell sorting (FACSAria II, BD Biosciences). After separation, contaminating $MOLT_{NL4-3}$ cells (<1%) were assessed by FSC/SSC parameters using flow cytometry. The possible contribution to infection of persisting $MOLT_{NL4-3}$ cells (<1%) was evaluated using the coculture performed with the mAb IgGb12, a condition where HIV-1 uptake into $CD4^+$ T cells is blocked, and therefore, infection of purified $CD4^+$ T cells would only come from remaining $MOLT_{NL4-3}$ cells.

Culture of HIV-1-loaded Cells—Isolated $CD4^+$ T cells from each initial coculture condition were subdivided in three and cultured for 5 days in the following medium conditions: (i) 80 nM mAb IgGb12; (ii) 80 nM mAb IgGb12 and 4 μM RT inhibitor AZT; or (iii) left untreated. $CD4^+$ T cells were activated by adding 4 μg/ml PHA and 6 units/ml IL-2 to the medium. After 5 days, infection in target cells was assessed by enzyme-linked immunosorbent assay (ELISA) for HIV-CAp24 antigen detection in culture supernatants (Genscreen HIV-1 Ag EIA; Bio-Rad Laboratories).

Determination of Anti-HIV Activity in Cell-free Virus Infections and Cell-Cell Transfer—The anti-HIV activity using cell-free virus infections was determined as described before (27). Briefly, PHA-activated $CD4^+$ T lymphocytes (1.5×10^5 cells/well) were incubated with $HIV\text{-}1_{NL4-3}$ ($200\ TCID_{50}/10^6$ cells) or mock-infected during 7 days at 37 °C, 5% CO_2 in the presence of different concentrations of the corresponding test compound. HIV-1 CAp24 antigen production in the supernatant was measured by a commercial ELISA test as described above. To determine cytotoxicity, mock-infected cells were harvested and fixed with 1% formaldehyde. Cell death was quantified by flow cytometry in forward *versus* side scatter plots. Dead cells showed increased side and reduced forward scatter values compared with those of living cells. Anti-HIV activities were determined in at least three independent experiments, performed in triplicate. To evaluate the anti-HIV activity in cell-cell transfer, overnight cocultures between isolated primary $CD4^+$ T cells (2×10^5) and uninfected or infected $MOLT_{NL4-3}$ cells (2×10^5) were performed in the presence of serial dilutions of the corresponding test compounds. Virus transfer was measured as described above. The 50% effective concentration (EC_{50}) and the 50% cytotoxic concentration (CC_{50}) were calculated for cell free-virus infections and cell-cell CAp24 antigen transfer. Bafilomycin A1 (BFLA1) and concanamycin A (CON A) were purchased from Sigma.

Infection with Viruses Released from Antigen-loaded Cells—Cocultures between freshly isolated primary $CD4^+$ T cells and uninfected or infected $MOLT_{NL4-3}$ cells were performed as described above. After 6 h of coculture, to minimize the possibility of $CD4^+$ T cell infection, target cells were sorted (>99% purity) as indicated above, and recovered target cells were cultured (5×10^5 cells/condition) in the presence or the absence of 80 nM IgGb12 to prevent productive infection. After 12 h of culture, the presence of CAp24-antigen was evaluated both in the supernatant and in the purified cells by intracellular CAp24 antigen staining as indicated above. Total viral DNA was also quantified by PCR as indicated below using infected $CD4^+$ T

cells as a positive control. For each condition, 20 μl of supernatant was used to infect 3×10^4 MT4 cells for 5 days. Infection of MT4 T cells was evaluated by quantification of supernatant CAp24-antigen content.

Quantitative Real-time PCR for Total HIV-1 DNA Detection—Total DNA was quantified as described before (28, 29). Briefly, purified $CD4^+$ T lymphocytes were centrifuged, supernatant was removed, and pellets were frozen. Total cellular DNA was extracted using QIAamp DNA extraction kit (QIAamp DNA Blood mini kit; Qiagen) as recommended by the manufacturer. Quantitative amplification of LTR for viral entry detection was performed using the following primers and probe (forward, 5′-GACGCAGGACTCGGCTTG-3′; reverse, 5′-ACTGACGCTCTCGCACCC-3′ and probe 5′-TTTGGCGTACTCACCAGTCGCCG-3′ labeled with the fluorophore FAM and the quencher TAMRA). To normalize HIV copy values/cell, amplification of cellular RNaseP gene was performed using TaqMan® RNaseP Control Reagents Kit (Applied Biosystems). DNA extracted from 8E5/LAV cells (harboring one copy of integrated HIV-1/cell) was used to build a standard curve. The PCR was performed in a total volume of 50 μl using 1× TaqMan® Universal PCR Master Mix (Applied Biosystems, Roche), 0.9 μM concentration of the primers, 0.25 μM probe, and 5 μl of the DNA sample. Reactions were analyzed with the ABI PRISM 7000 instrument using SDS 1.1 software (Applied Biosystems). For each condition, the amount of the total viral DNA/cell was normalized to untreated sample with IgGb12, and results are expressed as the relative percent increase.

Virus-Cell Fusion Assay—The quantification of the virus-cell membrane fusion was quantified as described before (30). Briefly, 1×10^5 HEK293-T cells were cotransfected with 0.4 μg of both the NL4-3 HIV provirus plasmid (pNL4-3 from the AIDS Reagents Program) and a plasmid carrying the *Vpr* gene fused with β-lactamase (*Vpr*-BlaM; pMM310 from the AIDS Reagents Program). After 48 h, transfected HEK293-T cells were cocultured overnight with primary $CD4^+$ T lymphocytes as described above. Cells were then recovered and loaded with the CCF2-AM loading kit (Invitrogen) following the protocol provided by the manufacturer. Cells were incubated for 1 h at room temperature, then washed and immediately fixed. The change in emission of the cleaved CCF2 generated by the *Vpr*-BlaM chimera was measured by flow cytometry.

Evaluation of Dynasore Activity—Primary $CD4^+$ T lymphocytes were pretreated with or without different concentrations of dynasore starting at 160 μM, for 30 min at 37 °C. Then, pretreated $CD4^+$ T lymphocytes were cultured in the presence or the absence of phorbol 12-myristate 13-acetate (PMA; Sigma) at 1 μg/ml for 30 min at 37 °C. Cells were fixed with 1% formaldehyde and after washes with PBS, stained for CD4 expression with anti-CD4 mAb conjugated with the fluorochrome FITC (BD Bioscience). Analysis of cells was performed by flow cytometry.

Immunofluorescence, Confocal Microscopy, and Quantification of Colocalization—For immunofluorescence staining, cocultures of primary $CD4^+$ T cells with uninfected or infected $MOLT_{NL4-3}$ cells were performed as described above. Samples were trypsinized to remove potentially bound viruses into the cell surface and after subsequent washes with PBS cells were fixed, permeabilized (Fix & Perm), and incubated for 1 h at room temperature with the anti-CAp24 mAb KC57-FITC (Coulter) and the CD4-PE (BD Bioscience) or with the goat anti-human-dynamin antibody (clone N-19, Santa Cruz Biotechnology). For dynamin staining, cells were then washed and incubated for 1 h at room temperature with the donkey anti-goat Alexa Fluor 647-conjugated secondary antibody (Molecular Probes, Invitrogen). Cells were adhered onto glass slides using cytospins (Thermo Scientific) and mounted with Prolong Gold antifade reagent (Invitrogen). Images were acquired on a Leica TCS SP5 AOBS confocal microscope (Leica Microsystems CMS GmbH, Mannheim, Germany). Z-sections were acquired at 0.5-μm steps using an Argon 488/458 and HeNe 633 lasers and a plan Apochromat 63 × 1.4 oil objective, supplied with the imaging software LAS AF (Leica Microsystems). Determination of the colocalization coefficient between CAp24 protein and the CD4 receptor or dynamin protein was performed using single Z-stacks and evaluated with LAS AF software.

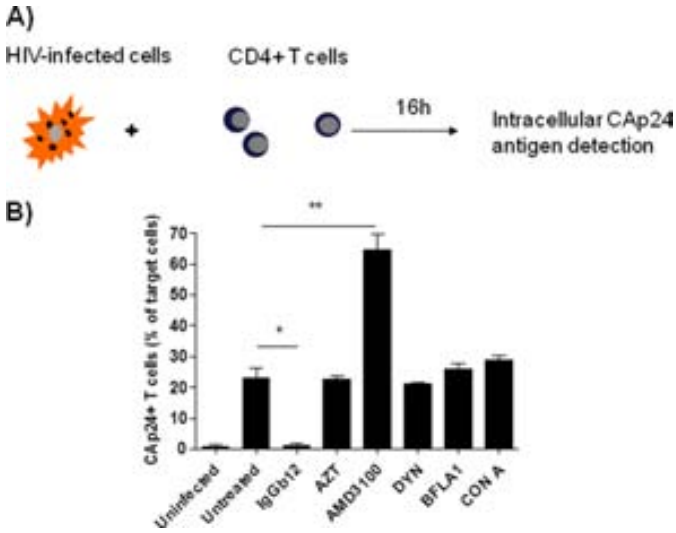


FIGURE 1. **CD4-dependent transfer of HIV antigen after cell-to-cell contacts.** *A*, experimental procedure was overnight cocultures of $MOLT_{NL4-3}$ cells with primary $CD4^+$ T lymphocytes. *B*, cocultures were performed in the presence of 80 nM anti-gp120 mAb IgGb12; 4 μM RT inhibitor AZT; 12.5 μM CXCR4 antagonist AMD3100; 80 μM dynamin inhibitor dynasore (*DYN*); 100 nM BFLA1, and 20 nM CON A. Results are represented as the percentage of intracellular $CAp24^+$ target cells, using the coculture between $CD4^+$ T cells and uninfected MOLT cells as a negative control. Results are the mean ± S.D. (*error bars*) of three independent experiments (**, $p < 0.005$; *, $p < 0.05$).

Statistical Analysis—Student's *t* test was used to determine statistical significance (**, $p < 0.005$ or *, $p < 0.05$) between values.

RESULTS

HIV Transmission during Cell-to-cell Cocultures—Overnight cocultures of HIV-1 NL4-3 persistently infected MOLT-4/CCR5 cells ($MOLT_{NL4-3}$) with CMFDA-loaded nonstimulated primary $CD4^+$ T lymphocytes were evaluated by flow cytometry. After overnight coculture, intracellular staining of capsid p24 (CAp24) HIV antigen was detected in 23% of target cells (Fig. 1). The transfer of viral antigens to uninfected cells was clearly blocked by the neutralizing anti-gp120 mAb IgGb12 (95% of inhibition compared with the untreated condition), but

TABLE 1
Potent postattachment inhibitors of HIV replication do not block cell-to-cell transfer of virus

Compound	Anti-HIV-1 activity		Cell-to-cell HIV-1 Transfer	
	EC_{50} [a]	CC_{50} [b]	EC_{50} [c]	CC_{50} [d]
	μM	μM	μM	μM
AMD3100	0.018 ± 0.0023	>0.125	No effect at 62 μM	>62
AZT	0.0021 ± 0.0003	>0.4	No effect at 20 μM	>20
IgGb12	0.0003 ± 0.0001	>0.04	0.0006 ± 0.0001	>0.4
Dynasore	No effect at 40 μM	40	No effect at 80 μM	250

[a] EC_{50}: Effective concentration needed to inhibit 50% replication of the wild-type HIV-1NL4–3 strain in peripheral blood mononuclear cells.
[b] CC_{50}: Cytotoxic concentration needed to induce 50% death of noninfected cells, evaluated by morphology changes using flow cytometry 7 days after infection.
[c] EC_{50}: Effective concentration needed to block 50% of HIV-1NL4-3-antigen transfer in $CD4^+$ T cells determined by intracellular CAp24 antigen staining after overnight cocultures between HIV-infected $MOLT_{NL4-3}$ cells and primary $CD4^+$ T cells.
[d] CC_{50} evaluated after overnight cocultures.

was not inhibited by the RT inhibitor AZT or the dynamin-dependent endosome-scission inhibitor dynasore (21 and 22% of $p24^+$ cells, respectively) despite using high drug concentrations (2000-fold higher than the EC_{50} of AZT under cell-free infection conditions, Table 1). Macrolide antibiotics such as BFLA1 and CON A that prevent endosome and lysosome acidification did not have any effect on virus uptake (Fig. 1*B*).

Interestingly, in the presence of the coreceptor antagonist AMD3100 the uptake of HIV particles by the target cells increased roughly 3-fold compared with untreated condition (65% of target cells were positive for CAp24 antigen staining) even when cells were cocultured with ~700-fold higher EC_{50} (Table 1). Taken together, these results confirmed that cellular contacts between infected lymphoid cells and primary $CD4^+$ T lymphocytes triggered CD4-dependent transmission of high amounts of HIV-1 particles from infected to uninfected cells.

To evaluate virus-cell fusion, HIV-1 NL4-3 transfected *Vpr*-$BlaM^+$ HEK293-T cells were cocultured with target $CD4^+$ T cells and fusion was measured by detection of cleaved CCF2. As expected, mAb IgGb12 completely blocked virus-dependent fusion similar to the observed inhibition of virus capture (Fig. 2). Conversely, AMD3100 blocked virus-cell fusion (Fig. 2) although it did not block but significantly increased virus transfer (Fig. 1). AZT or dynasore did not prevent cleavage of CCF2, suggesting that virus antigen was passively transferred to $CD4^+$ T cells in the absence of virus cell fusion as noted in the AMD3100-treated cells.

Productive Infection Did Not Occur from within Intracellular Compartments—We hypothesize that internalized virus after cell-to-cell transfer could not fuse from within intracellular compartments. To evaluate the fate of internalized HIV-1 particles captured by $CD4^+$ T cells after cell-to-cell transfer, CMFDA-loaded target $CD4^+$ T cells were purified from infected $MOLT_{NL4-3}$ lymphoid cells by fluorescence-activated cell sorting (>99% purity). Following separation, purified $CD4^+$ T cells were trypsinized to eliminate virus bound to the cell surface. Trypsin treatment dramatically reduced the expression of CD4 in purified T cells; however, CAp24 antigen staining was not significantly reduced (data not shown), suggesting that captured virus resided in intracellular compartments. Immediately after washings, for each initial coculture condition, target cells were subdivided in three and left in culture during 5 days in drug-free medium or in the presence of the mAb IgGb12 or IgGb12+AZT (Fig. 3*A*). Drug concentrations used clearly ensure complete inhibition of infection (300-fold and 2000-fold higher EC_{50} for IgGb12 and AZT, respectively, Table 1). Dynasore was not included as it was cytotoxic in long term cultures (data not shown). Virus production is low to undetectable in nonstimulated cells (18, 31, 32); thus, PHA/IL-2 was added to the medium to promote virus replication. After 5 days in culture, CAp24 antigen in cell supernatant (Fig. 3*B*) and total viral DNA detection by quantitative PCR (Fig. 3*C*) were evaluated as a measure of virus replication and indicated that antigen-loaded cell cultures became productively infected after PHA/IL-2 activation in the absence of inhibitors in the culture medium (Fig. 3, *gray bars*). Virus production was in concordance to the amount of virus transferred during the coculture phase (Fig. 1). Thus, in the absence of antigen transfer (IgGb12-treated coculture), no virus production was found. Conversely, the high uptake of CAp24-antigen in the AMD3100-treated cocultures coincided with an increase in virus production in purified cells. The RT inhibitor AZT did not prevent virus transfer or fusion and partially blocked supernatant CAp24 antigen production or total DNA detection as a consequence of being present only during the coculture phase. However, when IgGb12 was present during the purified cell

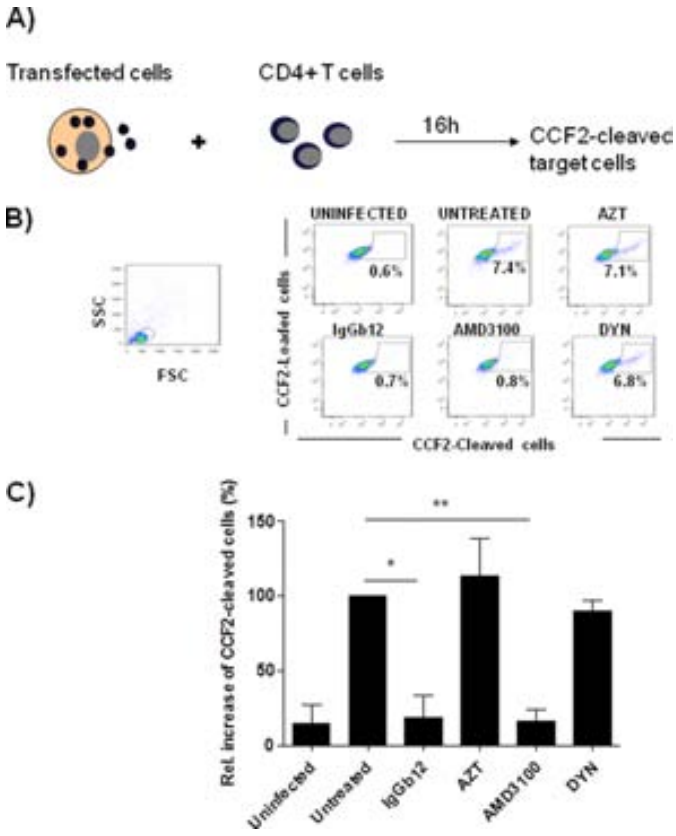


FIGURE 2. **IgGb12 and AMD3100 but not dynasore blocked virus-cell fusion after cell to cell transfer of virus.** *A*, experimental procedure: measurement of viral fusion in cocultures of HEK293-T cells transfected with pNL4-3 and *Vpr*-BlaM plasmids and primary $CD4^+$ T cells. *B*, dot plots of CCF2-loaded cells (FITC-labeled) *versus* CCF2-cleaved cells (*Pacific blue*-labeled). A representative experiment is shown. *C*, relative increase of CCF2-cleaved target cells compared with untreated condition. Data are the mean ± S.D. (*error bars*) of three independent experiments (**, $p < 0.005$; *, $p < 0.05$) (*DYN*, dynasore).

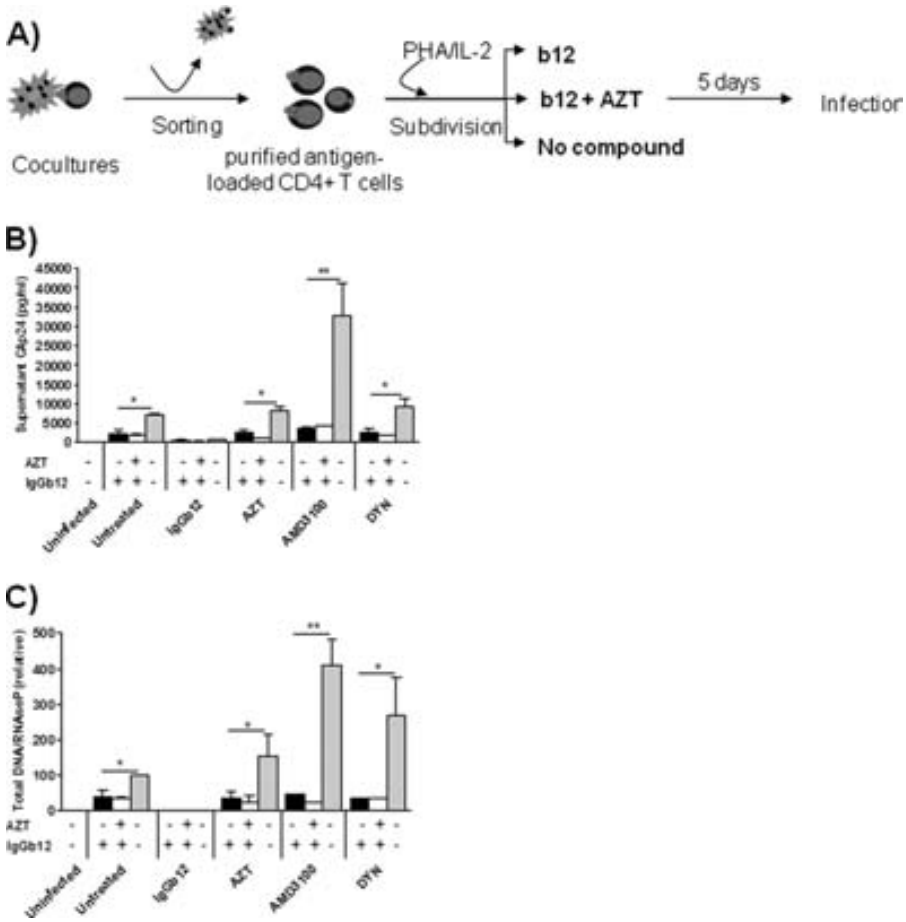


FIGURE 3. **Infection of CD4$^+$ T cells by HIV particles captured into trypsin-resistant compartments was inhibited by mAb IgGb12.** *A*, experimental procedure: isolation and culture of CAp24-loaded CD4$^+$ T cells. After 5 days of culture, HIV infection was assessed by supernatant CAp24 antigen production, expressed in pg/ml (*B*) and quantification of total viral DNA as the copy number of total DNA/RNaseP, expressed relative to the untreated condition (cells untreated during the coculture and culture phase) (*C*). Results represent the mean ± S.D. (*error bars*) of three independent experiments (**, $p < 0.005$; *, $p < 0.05$) (*DYN*, dynasore).

culture phase (Fig. 3, *black* and *white bars*), virus replication was significantly blocked irrespective of the condition used during the coculture phase (Fig. 3, *x* axis, *angled labels*). Taken together, these results indicate that the conditioned medium with IgGb12 prevented internalized virus particles from initiating a productive infection. Virus needed to reach the extracellular environment to initiate a productive infection, an event that could only occur when the attachment inhibitor, mAb IgGb12, was not present.

Infection by HIV Particles Released from Antigen-loaded Cells—Our results suggest that the inability of virions to infect cells from within endosomal compartments could promote the recycling of HIV particles to the cell surface that could later infect bystander cells. To further explore this hypothesis, antigen-loaded primary CD4$^+$ T lymphocytes were sorted after short term cocultures (6 h) with infected MOLT$_{NL4-3}$ cells (Fig. 4*A*). IgGb12-treated coculture, in which CAp24-antigen transfer was completely blocked, was used to control the effect of contaminant MOLT$_{NL4-3}$ cells (<0.1%). Once purified, antigen-loaded CD4$^+$ T cells were left in culture for 12 h in the presence or the absence of IgGb12 to restrict reinfection events while allowing release of virions in the supernatant. The CAp24-antigen found in the supernatant was concordant with the level of intracellular CAp24-antigen in loaded target cells (Fig. 4*B*). Total DNA in purified target cells was measured to ensure that antigen-loaded cells did not become infected during the culture (Fig. 4*C*). Compared with infected control cells, target cells remained negative, suggesting that particles found in the supernatant did not come from new infection events but released from endocytic compartments. Supernatants were collected after 12 h and used to infect lymphoid MT4 T cells (Fig. 4*D*). The supernatants from untreated and AMD3100-treated cultures were able to establish a productive infection in MT4 cells. Conversely, the supernatant of the IgGb12 condition could not infect target cells, indicating that infection was not generated from contamintant MOLT$_{NL4-3}$ cells. These results indicate that antigen-loaded cells did not become infected but were able to infect bystander CD4$^+$ cells after recycling of HIV to the cell surface and release to the cell supernatant.

Dynasore Did Not Block Uptake or Infection of CD4$^+$ T Cells—Dynasore (80 μM), a dynamin-dependent endosomal scission inhibitor, has been shown to block the infection of HeLa cells, suggesting that endosomal uptake was a prerequisite for fusion and infection (20). We and others have shown that cell-to-cell transfer of HIV may occur through an endocytic process in which virus antigen is colocalized with clathrin and dynamin

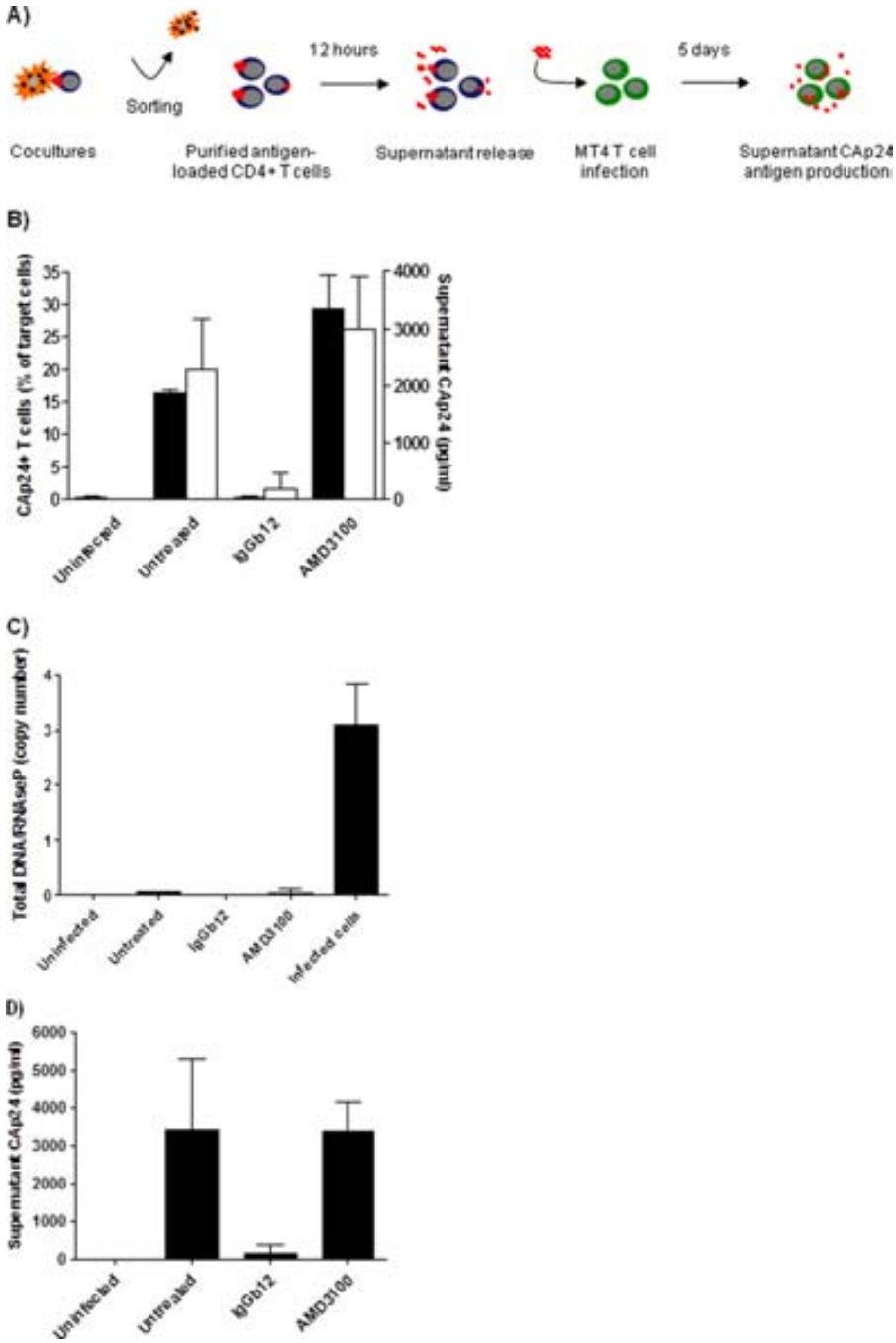


FIGURE 4. **Trans-infection by released HIV viruses from antigen-loaded cells.** *A*, in this experimental procedure, supernatants from cocultures were collected and used to infect MT4 T cells. *B*, after 12 h of culture, p24-antigen content was evaluated in the supernatant (*white bars*) and in the purified cells (*black bars*) by CAp24 ELISA and intracellular CAp24 antigen staining, respectively. *C*, total viral DNA was also quantified in purified cells by PCR using infected $CD4^+$ T cells as a positive control. Results represent the total viral DNA copy number relative to the cellular control gene RNaseP. *D*, infection of MT4 T cells by collected supernatants was evaluated at day 5 by supernatant CAp24-antigen content. Data are the mean ± S.D. (*error bars*) of three independent experiments.

(12, 18, 19, 21, 33). However, dynasore did not prevent the CD4-dependent uptake of HIV antigen into target cells (Fig. 1), did not prevent virus replication in antigen-loaded, activated cells after cell-to-cell transfer of virus (Fig. 3), and was devoid of antiviral activity in peripheral blood mononuclear cells at subtoxic concentrations (Table 1). Conversely, dynasore blocked the PMA-induced down-regulation of the CD4 receptor in primary $CD4^+$ lymphocytes (Fig. 5), a process that involves a clathrin-dependent endocytic pathway (34).

To analyze the effect of dynasore in dynamin function during HIV uptake we performed a colocalization analysis between HIV Gag antigen (CAp24) and dynamin in untreated or dynasore-treated cocultures (Fig. 6*A*). Colocalization coefficients of 0.73 and 0.75 between CAp24 antigen and dynamin protein were calculated in both untreated and dynasore-treated cocultures respectively, indicating that early compartmentalization of HIV particles was associated with the dynamin endocytic machinery, but could not be blocked by an agent targeting the

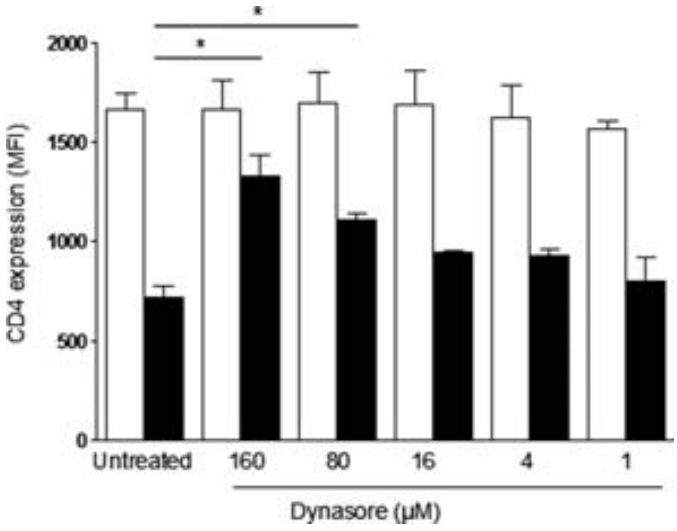


FIGURE 5. **Dynasore prevents PMA-induced down-regulation of CD4 receptor.** Primary $CD4^+$ T lymphocytes were pretreated for 30 min with or without 160, 80, 16, 4, and 1 μM of dynasore and then cultured in the absence (*white bars*) or the presence of PMA at 1 μg/ml (*black bars*) for an additional 30 min. Then, cells were fixed with 2% of formaldehyde, and surface CD4 expression (mean fluorescence intensity, *MFI*) was evaluated with an anti-CD4 mAb. Cells were analyzed by flow cytometry and identified by morphology. Dynasore inhibited PMA-induced CD4 down-regulation in a dose-dependent manner. Data are the mean ± S.D. (*error bars*) of three independent experiments. *, $p < 0.05$.

scission of early formed endosomes. Colocalization between HIV Gag antigen (CAp24) and CD4 receptor in untreated or dynasore-treated cocultures between primary $CD4^+$ T cells and infected $MOLT_{NL4-3}$ cells showed similar colocalization coefficients (0.78 and 0.84 for untreated and dynasore-treated conditions, respectively) (Fig. 6*B*).

DISCUSSION

Complete fusion of HIV particles within endosomal membranes has been used to indicate that internalization of HIV particles through an endocytic pathway was required for infection (20, 24). Here, we show, using primary CD4 T lymphocytes, that cell-to-cell contacts between HIV-infected and uninfected cells induced the endocytic uptake of viral particles into trypsin-resistant, dynamin-enriched compartments. Only the inhibition of gp120-CD4 interaction (virus attachment to CD4) could block the transfer of HIV particles. Conversely, the addition of the coreceptor inhibitor AMD3100 induced the accumulation of virus particles leading to massive endocytosis into cells in which the virus-cell fusion process was completely arrested (18, 25, 33). Activation of purified antigen-loaded cells initiated a productive infection but only when cells were cultured in the absence of mAb IgGb12, an inhibitor of virus attachment to CD4. IgGb12 should be unable to penetrate the cell surface. However, we cannot completely exclude the possibility of an antibody such as IgGb12 to enter already formed intracellular compartments containing HIV particles.

These results suggest that endocytosed viral particles could not initiate a productive infection from within endosomes in primary $CD4^+$ T cells (*i.e.* by virus fusion to the endosomal membrane). We hypothesize that endocytosed viruses could only induced infection in *trans* (trans-infection) because they were required to resurface and reach the extracellular environment and engage CD4 leading to virus-cell fusion and replication, a condition that could only be achieved in the absence of an attachment inhibitor in the cell supernatant. We have shown that antigen-loaded cells may release virus particles (11), and cocultures of antigen-loaded T cell with U87-CD4 target cells may lead to infection of the U87-CD4 cells (18), indicating the possibility of trans-infection. Here, we demonstrate that supernatant from purified antigen-loaded, but viral DNA-negative T cells, released virus to the supernatant that later infected MT4 cells, strongly suggesting that antigen-loaded cells trans-infect bystander $CD4^+$ T cells.

Recent data indicate that prior to membrane fusion, virions may need to undergo maturation after cell-to-cell transfer of HIV-1 (25), a process that might be impaired or further delayed in nonstimulated primary $CD4^+$ T cells, and thus, productive infection was only possible after virus recycling to the cell surface. Moreover, virion maturation may allow the virus to transfer from cell-to-cell in a conformation immunologically distinct that might escape the detection by neutralizing antibodies. However, these findings are in contrast to data showing that anti-gp41 antibodies 4E10 and 2F5 did not block the transfer of HIV particles from infected to target cells but blocked productive infection of target cells (35), suggesting that HIV infection between T cells is transmitted by a neutralization-sensitive mechanism (4, 35). Our results reinforce the idea that endocytosed virus after cell-to-cell contacts may represent an itinerant virus reservoir able to induce the trans-infection of bystander T cells, but not leading to effective virus fusion or replication from within internal endosomal compartments. The contribution of this mechanism in the pathogenesis of HIV *in vivo* still needs to be completely clarified but should be taken into account when developing new antiviral strategies (36).

Using confocal microscopy, we found clathrin and dynamin proteins colocalized with HIV particles (33) which in turn were colocalized with CD4 (Fig. 6). However, dynasore, a dynamin-dependent endosomal scission inhibitor previously shown to block virus replication in HeLa cells (20, 24), did not prevent virus capture, virus cell fusion, and virus replication after cell-to-cell transfer to primary $CD4^+$ T cells. In concordance, previous observations indicated that VSV-G-pseudotyped HIV infection could not be inhibited after dynasore treatment, suggesting that VSV and HIV envelopes mediate distinct modes of virus entry (37). Moreover, it has been demonstrated that dynasore inhibits clathrin-mediated endocytosis at two different steps. The ultrastructural analysis of the effect of dynasore on clathrin-coated structures shows the appearance of "U" and "O" shape-coated pits associated with the plasma membrane (38). Consequently, internalization of CAp24 antigen into the "initial" coated pits in the presence of dynasore cannot be ruled out. Altogether, it appears that internalization of particles initially required the endocytic machinery, and dynasore might not be able to inhibit the initial formation of these endocytic compartments. Blocking HIV endocytosis (*e.g.* with dynasore) without preventing virus replication would be the ultimate proof of endocytosis not being necessary for infection. This could not be achieved with dynasore at nontoxic concentrations, and there-

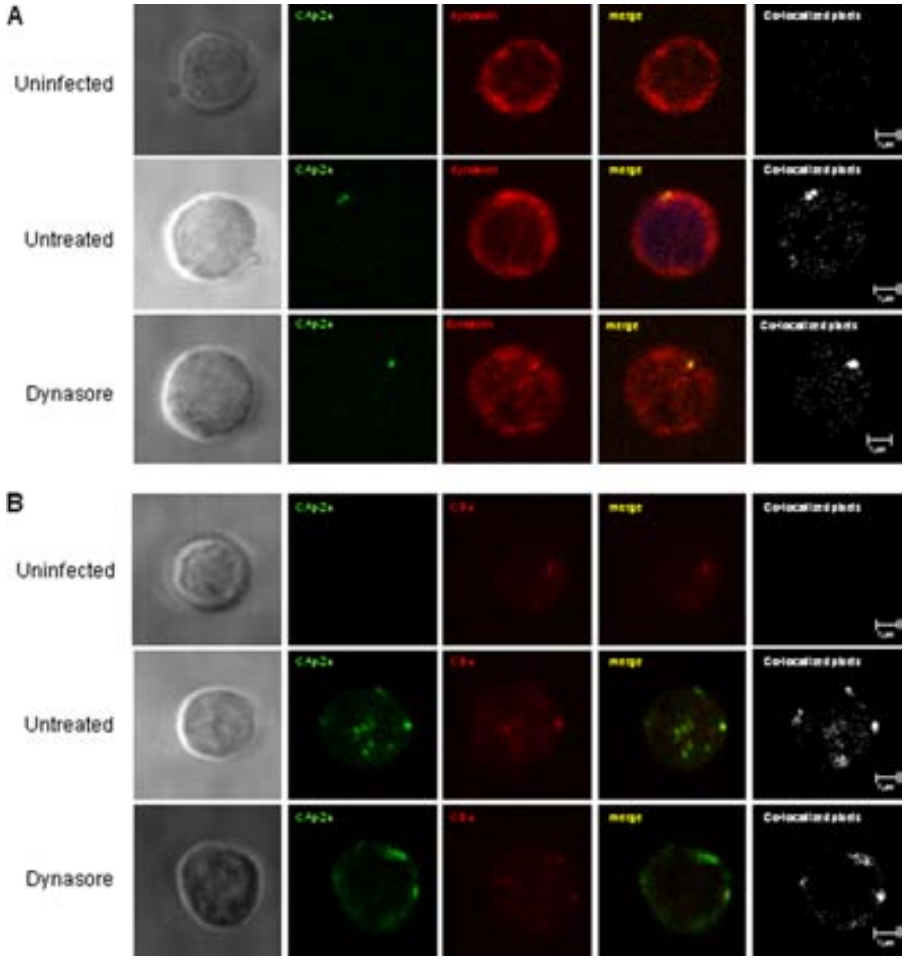


FIGURE 6. **Uptake of HIV particles into intracellular CD4$^+$ compartments in primary T lymphocytes was not blocked by dynasore.** Primary CD4$^+$ T lymphocytes were cocultured overnight with HIV-infected MOLT$_{NL4-3}$ cells in the presence or the absence of dynasore (80 μM). Recovered cells were trypsinized to remove membrane-bound viruses and immunostained with antibodies against HIV CAp24 antigen, CD4 receptor, and dynamin. Sections of single target CD4$^+$ T cells were viewed and analyzed by confocal microscopy. Colocalization between (*A*) HIVp24 antigen (*green*) and dynamin protein (*red*) or between (*B*) HIVp24 antigen (*green*) and CD4 receptor (*red*) was performed for uninfected (*upper panels*), untreated (*middle panels*), and dynasore-treated (*lower panels*) cocultures. The images show the phase-contrast (*left column*), the single stainings, the overlay (*yellow*), and the colocalized pixels (*white*). A CD4$^+$ T lymphocyte representative of each coculture is shown from at least two independent experiments.

fore, the hypothesis remains unresolved. However, we clearly show that internalized virus required to resurface to initiate a productive infection, suggesting that endocytosis may not be a route of productive infection.

The development of new small molecule inhibitors of clathrin-coated pit assembly (Pitstop) allowed better characterization of clathrin functions within the endocytic network (39). Pitstop-induced inhibition of clathrin terminal domain interferes with receptor-mediated endocytosis and synaptic vesicle recycling and has been shown to increase the lifetime of clathrin-coated components, including dynamin. These agents were also shown to block HIV entry in HeLa cells, but it remains to be resolved whether inhibition of virus replication was due to preventing virus-endosome fusion or the recycling of HIV particles. Importantly, the antiviral activity of endosome function should be evaluated in primary T cells that better model the interactions between virus and cell functions.

Nef-induced down-regulation of CD4 results in internalization and degradation of surface CD4 in lysosomes (40). Prevention of endosome and lysosome acidification by macrolide antibiotics such as BFLA1 and CON A inhibits degradation of CD4 and consequently promotes accumulation of CD4 in endosomes and lysosomes (41). Moreover, different types of endosome acidification inhibitors increase infectivity of HIV particles presumably by preventing them from degradation in late endosomes and lysosomes (42). Colocalization of HIV particles with CD4 in dynamin-containing endosomes could indicate that CD4 protects virus particles from degradation and helps recycle back HIV to the cell surface. However, in our hands, acidification inhibitors such as BFLA1 and CON A did not prevent or augment virus transfer from infected to uninfected cells. Therefore, our results did not shed light on the protective role of CD4 in endocytic virus degradation.

In conclusion, after cell-to-cell transfer of HIV-1 into target primary CD4$^+$ T cells we observed that cells were only infected if left in culture in the absence of an attachment inhibitor to CD4 (mAb IgGb12), suggesting that virus needed to resurface to begin a productive infection. Moreover, dynasore, an inhibitor of dynamin-dependent endocytosis, did not block virus replication. Endocytosis may not be the primary mechanism of infection by HIV-1 after cell-to-cell contact, but a reservoir able to induce trans-infection of bystander CD4$^+$ T cells.

Acknowledgments—We thank the National Institutes of Health (AIDS Research and Reference Reagent Program) and the EU Programme EVA Centralised Facility for AIDS Reagents, National Institute for Biological Standards and Control, United Kingdom, for reagents. We thank Marco A. Fernández (Instituto Germans Trias i Pujol, Badalona, Spain) for technical assistance.

REFERENCES

1. Adamson, C. S., and Freed, E. O. (2010) Novel approaches to inhibiting HIV-1 replication. *Antiviral Res.* **85,** 119–141
2. Marsh, M., and Helenius, A. (2006) Virus entry: open Sesame. *Cell* **124,** 729–740
3. Sattentau, Q. (2008) Avoiding the void: cell-to-cell spread of human viruses. *Nat. Rev. Microbiol.* **6,** 815–826
4. Martin, N., Welsch, S., Jolly, C., Briggs, J. A., Vaux, D., and Sattentau, Q. J. (2010) Virological synapse-mediated spread of human immunodeficiency virus type 1 between T cells is sensitive to entry inhibition. *J. Virol.* **84,** 3516–3527
5. Sigal, A., Kim, J. T., Balazs, A. B., Dekel, E., Mayo, A., Milo, R., and Baltimore, D. (2011) Cell-to-cell spread of HIV permits ongoing replication despite antiretroviral therapy. *Nature* **477,** 95–98
6. Izquierdo-Useros, N., Esteban, O., Rodriguez-Plata, M. T., Erkizia, I., Prado, J. G., Blanco, J., García-Parajo, M. F., and Martinez-Picado, J. (2011) Dynamic imaging of cell-free and cell-associated viral capture in mature dendritic cells. *Traffic* **12,** 1702–1713
7. Permanyer, M., Ballana, E., and Esté, J. A. (2010) Endocytosis of HIV. *Trends Microbiol.* **18,** 543–551
8. Jolly, C., and Sattentau, Q. J. (2004) Retroviral spread by induction of virological synapses. *Traffic* **5,** 643–650
9. Esté, J. A., and Telenti, A. (2007) HIV entry inhibitors. *Lancet* **370,** 81–88
10. Tilton, J. C., and Doms, R. W. (2010) Entry inhibitors in the treatment of HIV-1 infection. *Antiviral Res.* **85,** 91–100
11. Bosch, B., Blanco, J., Clotet-Codina, I., Pauls, E., Armand-Ugón, M., Grigorov, B., Muriaux, D., Clotet, B., Darlix, J. L., and Esté, J. A. (2005) Inhibition of coreceptor independent cell-to-cell HIV-1 transmission by a CD4-IgG2 fusion protein. *Antimicrob. Agents Chemother.* **49,** 4296–4304
12. Hübner, W., McNerney, G. P., Chen, P., Dale, B. M., Gordon, R. E., Chuang, F. Y., Li, X. D., Asmuth, D. M., Huser, T., and Chen, B. K. (2009) Quantitative 3D video microscopy of HIV transfer across T cell virological synapses. *Science* **323,** 1743–1747
13. Puigdomènech, I., Massanella, M., Izquierdo-Useros, N., Ruiz-Hernandez, R., Curriu, M., Bofill, M., Martinez-Picado, J., Juan, M., Clotet, B., and Blanco, J. (2008) HIV transfer between CD4 T cells does not require LFA-1 binding to ICAM-1 and is governed by the interaction of HIV envelope glycoprotein with CD4. *Retrovirology* **5,** 32
14. Stein, B. S., Gowda, S. D., Lifson, J. D., Penhallow, R. C., Bensch, K. G., and Engleman, E. G. (1987) pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. *Cell* **49,** 659–668
15. Bedinger, P., Moriarty, A., von Borstel, R. C., 2nd, Donovan, N. J., Steimer, K. S., and Littman, D. R. (1988) Internalization of the human immunodeficiency virus does not require the cytoplasmic domain of CD4. *Nature* **334,** 162–165
16. Kielian, M., and Jungerwirth, S. (1990) Mechanisms of enveloped virus entry into cells. *Mol. Biol. Med.* **7,** 17–31
17. Skehel, J. J., and Wiley, D. C. (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu. Rev. Biochem.* **69,** 531–569
18. Blanco, J., Bosch, B., Fernández-Figueras, M. T., Barretina, J., Clotet, B., and Esté, J. A. (2004) High level of coreceptor-independent HIV transfer induced by contacts between primary CD4 T cells. *J. Biol. Chem.* **279,** 51305–51314
19. Clotet-Codina, I., Bosch, B., Senserrich, J., Fernandez-Figueras, M. T., Pena, R., Ballana, E., Bofill, M., Clotet, B., and Este, J. A. (2009) HIV endocytosis after dendritic cell to T cell viral transfer leads to productive virus infection. *Antiviral Res.* **83,** 94–98
20. Miyauchi, K., Kim, Y., Latinovic, O., Morozov, V., and Melikyan, G. B. (2009) HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. *Cell* **137,** 433–444
21. Chen, P., Hübner, W., Spinelli, M. A., and Chen, B. K. (2007) Predominant mode of human immunodeficiency virus transfer between T cells is mediated by sustained Env-dependent neutralization-resistant virological synapses. *J. Virol.* **81,** 12582–12595
22. Pauza, C. D., and Price, T. M. (1988) Human immunodeficiency virus infection of T cells and monocytes proceeds via receptor-mediated endocytosis. *J. Cell Biol.* **107,** 959–968
23. Daecke, J., Fackler, O. T., Dittmar, M. T., and Kräusslich, H. G. (2005) Involvement of clathrin-mediated endocytosis in human immunodeficiency virus type 1 entry. *Journal of Virology* **79,** 1581–1594
24. de la Vega, M., Marin, M., Kondo, N., Miyauchi, K., Kim, Y., Epand, R. F., Epand, R. M., and Melikyan, G. B. (2011) Inhibition of HIV-1 endocytosis allows lipid mixing at the plasma membrane, but not complete fusion. *Retrovirology* **8,** 99
25. Dale, B. M., McNerney, G. P., Thompson, D. L., Hubner, W., de Los Reyes, K., Chuang, F. Y., Huser, T., and Chen, B. K. (2011) Cell-to-cell transfer of HIV-1 via virological synapses leads to endosomal virion maturation that activates viral membrane fusion. *Cell Host Microbe* **10,** 551–562
26. Sattentau, Q. (2010) Cell-to-cell spread of retroviruses. *Viruses* **2,** 1306–1321
27. Ballana, E., Pauls, E., Perron-Sierra, F., Clotet, B., Tucker, G. C., and Esté, J. A. (2011) β5 integrin is the major contributor to the αv integrin-mediated blockade of HIV-1 replication. *J. Immunol.* **186,** 464–470
28. Ballana, E., Pauls, E., Senserrich, J., Clotet, B., Perron-Sierra, F., Tucker, G. C., and Esté, J. A. (2009) Cell adhesion through αv-containing integrins is required for efficient HIV-1 infection in macrophages. *Blood* **113,** 1278–1286
29. Ballana, E., Senserrich, J., Pauls, E., Faner, R., Mercader, J. M., Uyttebroeck, F., Palou, E., Mena, M. P., Grau, E., Clotet, B., Ruiz, L., Telenti, A., Ciuffi, A., and Esté, J. A. (2010) Zinc ribbon domain-containing 1 (ZNRD1) is a host cellular factor influencing HIV-1 replication and disease progression. *Clin. Infect. Dis.* **50,** 1022–1032
30. Cavrois, M., De Noronha, C., and Greene, W. C. (2002) A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes. *Nat. Biotechnol.* **20,** 1151–1154
31. Doitsh, G., Cavrois, M., Lassen, K. G., Zepeda, O., Yang, Z., Santiago, M. L., Hebbeler, A. M., and Greene, W. C. (2010) Abortive HIV infection mediates CD4 T cell depletion and inflammation in human lymphoid tissue. *Cell* **143,** 789–801
32. Yoder, A., Yu, D., Dong, L., Iyer, S. R., Xu, X., Kelly, J., Liu, J., Wang, W., Vorster, P. J., Agulto, L., Stephany, D. A., Cooper, J. N., Marsh, J. W., and Wu, Y. (2008) HIV envelope-CXCR4 signaling activates cofilin to overcome cortical actin restriction in resting CD4 T cells. *Cell* **134,** 782–792
33. Bosch, B., Grigorov, B., Senserrich, J., Clotet, B., Darlix, J. L., Muriaux, D., and Esté, J. A. (2008) A clathrin-dynamin-dependent endocytic pathway for the uptake of HIV-1 by direct T cell-T cell transmission. *Antiviral Res.* **80,** 185–193
34. Pelchen-Matthews, A., Parsons, I. J., and Marsh, M. (1993) Phorbol ester-induced down-regulation of CD4 is a multistep process involving dissociation from p56lck, increased association with clathrin-coated pits, and altered endosomal sorting. *J. Exp. Med.* **178,** 1209–1222
35. Massanella, M., Puigdomènech, I., Cabrera, C., Fernandez-Figueras, M. T., Aucher, A., Gaibelet, G., Hudrisier, D., García, E., Bofill, M., Clotet, B., and Blanco, J. (2009) Antigp41 antibodies fail to block early events of virological synapses but inhibit HIV spread between T cells. *AIDS* **23,** 183–188
36. Permanyer, M., Ballana, E., Ruiz, A., Badia, R., Riveira-Munoz, E., Gonzalo, E., Clotet, B., and Esté, J. A. (2012) Antiretroviral agents effectively block HIV replication after cell to cell transfer. *J. Virology* **86,** 8773–8780
37. Yu, D., Wang, W., Yoder, A., Spear, M., and Wu, Y. (2009) The HIV envelope but not VSV glycoprotein is capable of mediating HIV latent infection of resting CD4 T cells. *PLoS Pathog.* **5,** e1000633
38. Macia, E., Ehrlich, M., Massol, R., Boucrot, E., Brunner, C., and Kirchhausen, T. (2006) Dynasore, a cell-permeable inhibitor of dynamin. *Dev. Cell* **10,** 839–850

39. von Kleist, L., Stahlschmidt, W., Bulut, H., Gromova, K., Puchkov, D., Robertson, M. J., MacGregor, K. A., Tomilin, N., Tomlin, N., Pechstein, A., Chau, N., Chircop, M., Sakoff, J., von Kries, J. P., Saenger, W., Kräusslich, H. G., Shupliakov, O., Robinson, P. J., McCluskey, A., and Haucke, V. (2011) Role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition. *Cell* **146,** 471–484
40. Rhee, S. S., and Marsh, J. W. (1994) Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. *J. Virol.* **68,** 5156–5163
41. Luo, T., Anderson, S. J., and Garcia, J. V. (1996) Inhibition of Nef- and phorbol ester-induced CD4 degradation by macrolide antibiotics. *J. Virol.* **70,** 1527–1534
42. Fredericksen, B. L., Wei, B. L., Yao, J., Luo, T., and Garcia, J. V. (2002) Inhibition of endosomal/lysosomal degradation increases the infectivity of human immunodeficiency virus. *J. Virol.* **76,** 11440–11446

JVI
Journals.ASM.org

Antiretroviral Agents Effectively Block HIV Replication after Cell-to-Cell Transfer

Marc Permanyer, Ester Ballana, Alba Ruiz, Roger Badia, Eva Riveira-Munoz, Encarna Gonzalo, Bonaventura Clotet, and José A. Esté

IrsiCaixa, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Spain

Cell-to-cell transmission of HIV has been proposed as a mechanism contributing to virus escape to the action of antiretrovirals and a mode of HIV persistence during antiretroviral therapy. Here, cocultures of infected HIV-1 cells with primary CD4$^+$ T cells or lymphoid cells were used to evaluate virus transmission and the effect of known antiretrovirals. Transfer of HIV antigen from infected to uninfected cells was resistant to the reverse transcriptase inhibitors (RTIs) zidovudine (AZT) and tenofovir, but was blocked by the attachment inhibitor IgGb12. However, quantitative measurement of viral DNA production demonstrated that all anti-HIV agents blocked virus replication with similar potency to cell-free virus infections. Cell-free and cell-associated infections were equally sensitive to inhibition of viral replication when HIV-1 long terminal repeat (LTR)-driven green fluorescent protein (GFP) expression in target cells was measured. However, detection of GFP by flow cytometry may incorrectly estimate the efficacy of antiretrovirals in cell-associated virus transmission, due to replication-independent Tat-mediated LTR transactivation as a consequence of cell-to-cell events that did not occur in short-term (48-h) cell-free virus infections. In conclusion, common markers of virus replication may not accurately correlate and measure infectivity or drug efficacy in cell-to-cell virus transmission. When accurately quantified, active drugs blocked proviral DNA and virus replication in cell-to-cell transmission, recapitulating the efficacy of antiretrovirals in cell-free virus infections and *in vivo*.

Antiretroviral therapy (ART) and human immunodeficiency virus research have achieved unprecedented series of breakthroughs that have translated into the largely successful management of what is now considered a chronic treatable infection (18, 33). In long-term-treated patients, the withdrawal of ART leads to a rebound of the plasma viral load, indicating that current treatment is unable to eradicate the virus from an infected patient despite apparently suppressed viral replication. Multiple factors may affect HIV persistence in the presence of antiretroviral treatment. Persistent viremia may arise from long-lived productively infected cells that were infected prior to therapy initiation or from the intermittent reactivation of latently infected cells. Alternatively, residual viral replication during ART or in sanctuary sites into which antiretrovirals poorly penetrate would explain HIV persistence (12). The degree to which HIV can effectively replicate during therapy is a highly contentious issue (14), and it is actively being studied in patients and cell culture models.

Recently, Sigal et al. (36) suggested that cell-to-cell transmission of HIV represents a mechanism contributing to virus escape from the action of antiretrovirals and a mode of HIV persistence during antiretroviral therapy. A mathematical model was used to demonstrate that the drug concentration required to prevent a single transmitted virion from infecting a target cell is much lower than that needed to stop multiple transmitted virus particles from infecting the same cell. Sigal et al. defined a transmission index (T_x) as the ratio between the number of infected cells in the presence of drug (I_d) and the fraction of cells infected in the absence of drug (I), adding further complexity to the model by associating T_x with the multiplicity of infection (MOI; abbreviated as "m" in the equation presented below), defined as the product of virus particle number and the infectivity per virus particle. Then, cell-to-cell spread was used as the experimental model to recapitulate the effect of multiple virus transmission.

We and others have demonstrated that coculture of HIV-1-infected cells with CD4$^+$ T cells leads to detection of large amounts of enveloped virions in clathrin-coated endosomes (6, 8, 9, 22, 27, 28, 31) that persist for 48 h (6) or as long as the target cells are cultured with infected cells. Cell-to-cell HIV transfer is blocked by agents that prevent virus attachment (7), but is resistant to agents targeting HIV coreceptors, gp41-dependent fusion inhibitors or neutralizing antibodies, and, importantly, agents targeting the reverse transcriptase (RT) (11, 25, 31). Endocytic transfer of HIV may lead to virus replication, but incoming viral antigen in target cells may incorrectly be interpreted as a marker of virus replication. Therefore, the aim of the present work was to evaluate the efficacy of known antiretrovirals in cell-associated virus transmission compared to cell-free virus infection. We show that anti-HIV drugs are able to block viral DNA production and the inherent virus replication in cell-to-cell viral transmission, recapitulating the efficacy of antiretrovirals in cell-free virus infection.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells (PBMCs) from healthy donors were purified by Ficoll-Hypaque sedimentation. CD4$^+$ T lymphocytes were immediately purified (>95%) from PBMCs by negative selection using the CD4$^+$ T cell enrichment kit (Stem Cell Technologies, Vancouver, Canada) and grown in RPMI 1640–L-glutamine medium (Gibco, Madrid, Spain). Chronically HIV-1-infected MOLT cells were generated after infection of the MOLT-4/CCR5 lymphoid cell line (AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, MD) with an NL4-3 X4 HIV-1 strain (HIV-1_{NL4-3}) constructed in an

Received 26 April 2012 Accepted 1 June 2012
Published ahead of print 13 June 2012
Address correspondence to José A. Esté, jaeste@irsicaixa.es.
Supplemental material for this article may be found at http://jvi.asm.org/.

doi:10.1128/JVI.01044-12

HIV_{HXB2} backbone (29). After the infection peak, the persistently infected culture was grown and characterized for Env expression and virus production (5). Uninfected MOLT-4/CCR5 cells were used as negative controls in all experiments. MOLT-4, the lymphoid T cell line MT-4 (obtained from the Medical Research Council, Centre for AIDS Reagents, London, United Kingdom), and the green fluorescent protein (GFP) expressing CEM-GFP cell line (AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, MD) were cultured in RPMI (Gibco, Madrid, Spain). Media were supplemented with 10% heat-inactivated fetal calf serum (FCS; Life Technologies, Madrid, Spain), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cocultures of infected and uninfected cells. Primary $CD4^+$ T cells were cocultured with uninfected or HIV-1 persistently infected MOLT cells as previously described (6, 7, 11). Briefly, target cells (purified primary $CD4^+$ T cells or MT-4 cells) were first labeled with the cell tracer DDAO-SE (Molecular Proves, Life Technologies) at a final concentration of 0.5 μg/ml for 30 min. Cells were extensively washed before being mixed with effector MOLT cells. Both effector and target cells (2×10^5 cells each in a 1:1 ratio) were cocultured in a 96-well culture plate in a final volume of 0.2 ml in the absence or the presence of the following HIV-1 inhibitors: the reverse transcriptase inhibitors (RTIs) zidovudine (AZT; 4 μM) (Sigma-Aldrich) and tenofovir disoproxil fumarate (TDF; 4 μM) (AIDS Research and Reference Reagent Program) and 10 μg/ml of the neutralizing anti-gp120 monoclonal antibody (MAb) IgGb12 (Polymun Scientific). After overnight coculture, primary $CD4^+$ T cells were purified (>99% purity) from MOLT cells by fluorescence-activated cell sorting (FACS) (FACSAria II; BD Biosciences). Recovered $CD4^+$ T cells were maintained in the presence of the same compound used during the initial coculture. After 5 days, infection in target cells was assessed by enzyme-linked immunosorbent assay (ELISA) for HIV capsid (CA) p24 antigen detection in culture supernatants (Genscreen HIV-1 Ag EIA; Bio-Rad Laboratories). Cocultures with target MT-4 T cells were performed during 2 h, 8 h, 24 h, and 48 h at 37°C.

Evaluation of HIV transfer. The capture of HIV-1 p24 antigen (p24) by primary $CD4^+$ T cells and MT-4 cells was evaluated by flow cytometry as shown before (8, 22). Prior to staining, cells were trypsinized to eliminate HIV-1 particles bound to the cell surface. For trypsin treatment, cells were washed with phosphate-buffered saline (PBS) and treated for 8 min at room temperature with 0.25% trypsin solution (Life Technologies, Madrid, Spain). Trypsin was stopped by addition of FCS, and cells were then washed with PBS. For intracellular staining, cells were fixed, permeabilized (Fix & Perm; Caltag, Burlingame, CA), and stained with the anti-HIV p24 antigen MAb KC57 (Coulter, Barcelona, Spain). Cells were analyzed in an LSRII flow cytometer (BD, Madrid, Spain) and identified by morphological parameters and DDAO-SE staining. Quantification of HIV-1 transfer was assessed by the percentage of p24-positive T cells, using the coculture between T lymphocytes and MOLT uninfected cells as a negative control.

Comparison of cell-free and cell-associated infections in CEM-GFP cells. CEM-GFP cells were infected either with cell-free virus or as a result of being cocultured with HIV-infected MOLT cells. For cell-free virus infections, 1×10^5 CEM-GFP cells were infected with 1,000 ng of an NL4-3 HIV strain (HIV_{NL4-3}) or mock infected with the same volume of medium. Coculture infections were performed at two different ratios of target to donor cells (2:1 and 9:1 CEM/MOLT ratio) and adjusted to a total of 2×10^5 cells. To differentiate effector from target cells by flow cytometry, MOLT cells were previously labeled with cell tracer DDAO-SE (Molecular Proves, Life Technologies) as described above. After extensive washes with PBS, 1.3×10^5 CEM-GFP cells were mixed with 0.6×10^5 MOLT cells at a 2:1 ratio and 1.8×10^5 CEM-GFP cells were mixed with 2×10^4 MOLT cells at a 9:1 ratio. For both ratios, cocultures with MOLT uninfected cells were performed as negative controls. Cell-free and cell-associated infections were carried out for 48 h in the presence or absence of various concentrations of AZT and TDF. Forty-eight hours postinfection, cells were recovered and infection was quantified by emission of green fluorescent protein (GFP) signal in target cells using flow cytometry in coculture infections. DDAO-SE and GFP double-positive cells, considered donor-target cell fusions, were excluded from the analysis. To quantify total GFP expression, cells were also viewed in a Nikon eclipse TE-200 microscope coupled to a charge-coupled device (CCD) Kappa camera, and the images obtained were used to quantify GFP expression. Quantification of total GFP was performed by evaluating and quantifying the average intensity of GFP signal for every image as implemented in the Launch VisionWorks software.

HIV-independent transactivation of HIV LTR in CEM-GFP cells. Cocultures between HeLa Env^+ cells (National Institute for Biological Standards and Control [NIBSC], United Kingdom) and CEM-GFP cells were performed to demonstrate unspecific replication-independent long terminal repeat (LTR)-driven expression of GFP in CEM-GFP cells (10, 19). A total of 2×10^5 HeLa Env^+ cells (also expressing Tat and Rev proteins) were seeded in a 24-well plate the day before the initiation of the coculture. After the removal of the supernatant, the coculture was initiated by the addition of 2×10^5 CEM-GFP cells in the presence or the absence of 4 μM tenofovir (TFV) in a final volume of 1 ml. Cocultures with the HeLa cell line TZM-bl (Env^-) were used as negative controls. Twenty-four hours later, cells were recovered and visualized in a Nikon eclipse TE-200 microscope coupled to a CCD Kappa camera.

Quantitative real-time PCR for proviral HIV-1 DNA detection. Proviral DNA was quantified as described before (3, 4). Briefly, cells were pelleted, supernatant was removed, and pellets were frozen at −20°C until use. Total cellular DNA was extracted using the QiaAmp DNA-extraction kit (QIAmp DNA blood minikit; Qiagen, Madrid, Spain) as recommended by the manufacturer. Quantitative amplification of LTR was performed using the following primers and probe (forward primer, 5′-GAC GCAGGACTCGGCTTG-3′; reverse primer, 5′-ACTGACGCTCTCGCA CCC-3′; probe, 5′-TTTGGCGTACTCACCAGTCGCCG-3′, labeled with the fluorophore 6-carboxyfluorescein [FAM] and the quencher 6-carboxytetramethylrhodamine [TAMRA]). To normalize HIV copy values per cell, amplification of the cellular RNase P gene was performed using the TaqMan RNase P control reagents kit (Applied Biosystems, Roche, Barcelona, Spain). For each experiment, DNA extracted from 8E5/LAV cells (harboring one copy of integrated HIV-1 per cell) was used to build a standard curve of the proviral DNA copy number, and DNA extracted from uninfected CEM-GFP cells was used to build a standard curve of the cell number. The PCR was performed in a total volume of 50 μl using 1× TaqMan Universal PCR master mix (Applied Biosystems), a 0.9 μM concentration of the primers, 0.25 μM probe, and 5 μl of the DNA sample. Reactions were analyzed with the ABI Prism 7000 instrument using SDS 1.1 software (Applied Biosystems). For each experiment with cell-associated infections, the background of proviral DNA copy number coming from MOLT infected cells was subtracted using a control condition in which an equal cell number of MOLT NL4-3 cells were cultured alone.

Calculation of T_x. The transmission index (Tx), designed to quantify the infection sensitivity to drugs (36), was calculated as the fraction of cells infected in the presence of drug (I_d) divided by the fraction of cells infected in the absence of drug (I). T_x depends on the multiplicity of infection (MOI) (symbolized here by the variable m), defined as a fraction of the number of infected cells by the number of target cells, and on the reduction of infection, $f(d)$, in a given drug concentration (d) as in the equation

$$T_x = \frac{I_d}{I} = \frac{1 - e^{-mf(d)}}{1 - e^{-m}}$$

In the present study, m corresponds to the percentage of infected cells (GFP^+ or $p24^+$) in the untreated condition, which was set to roughly 4% of GFP^+ cells under both cell-free and cell-associated infections. For each drug concentration tested, the T_x was calculated as the fraction of GFP^+ cells in the presence of drug by the percentage of GFP^+ cells in the absence of drug. T_x was equally calculated using the total HIV DNA or using the data obtained with the intracellular p24 antigen staining.

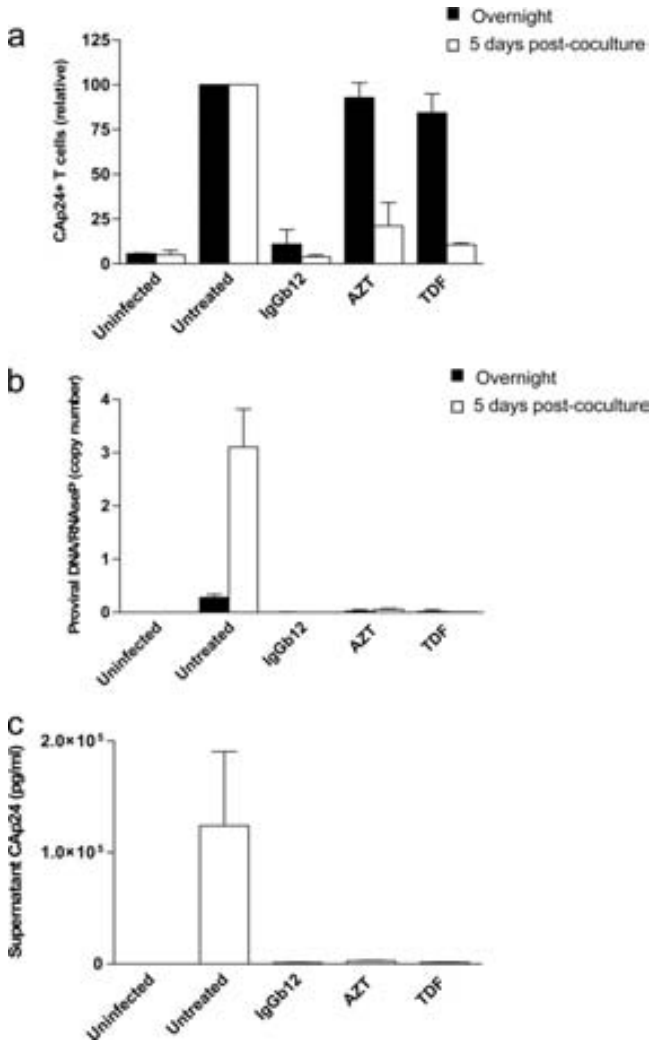


FIG 1 HIV antigen internalization in the absence of productive infection. Uninfected or HIV-1_{NL4-3}-infected MOLT cells were cocultured with primary $CD4^+$ T lymphocytes in the presence or the absence of IgGb12 (10 μg/ml), AZT (4 μM), and tenofovir (TDF, 4 μM). After overnight coculture, target cells were sorted and left in culture during 5 days in the presence of the same compound. Quantification of transferred HIV-1 antigen transfer was assessed by the percentage of intracellular HIV-1 p24 antigen-positive cells measured by flow cytometry and expressed relative to the untreated condition (a), and total viral DNA (proviral DNA) measured by qPCR and represented as the copy number of proviral DNA/cellular RNAse P copies (b) was assessed after overnight coculture (black bars) and 5 days post-coculture (white bars). Supernatant p24 antigen production (c) was also evaluated at day 5. The data shown are the means ± standard deviations (SD) of three independent experiments.

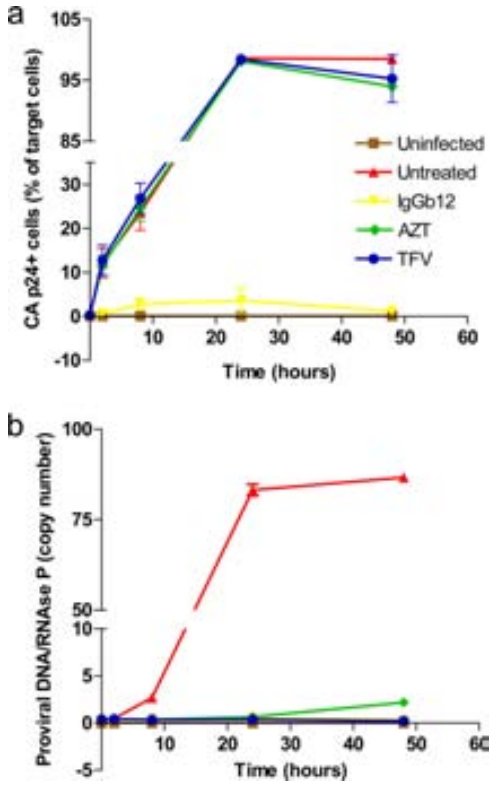


FIG 2 Virus transfer to lymphoid cells in the absence of virus replication. Uninfected or HIV-1_{NL4-3}-infected MOLT cells were cocultured with lymphoid $CD4^+$ MT-4 cells in the presence or absence of IgGb12 (10 μg/ml), AZT (4 μM), or TDF (4 μM). Two hours, 8 h, 24 h, and 48 h post-coculture, HIV-1 antigen transfer was assessed by the percentage of intracellular p24-positive cells using the coculture between MT-4 cells and MOLT uninfected cells as a negative control (a). Total viral DNA (proviral DNA), represented as the copy number of proviral DNA/cellular RNAse P copies, was used to quantify infection in target cells (b). The data shown are the means ± SD from three independent experiments.

RESULTS

Cell-to-cell transmission of HIV-1 in the absence of virus replication. We have previously shown that HIV-1 persistently infected or acutely infected T cells or dendritic cells may transfer HIV-1 particles to intracellular compartments in target $CD4^+$ T cells (6, 7, 11). After overnight cocultures of HIV-1_{NL4-3}-infected MOLT cells with nonstimulated primary $CD4^+$ T lymphocytes, roughly 20% of target cells were HIV antigen positive compared to the untreated condition (Fig. 1a, black bars). Antigen detection was resistant to the RT inhibitors AZT (4 μM) and TDF (4 μM), but was inhibited by the attachment inhibitor IgGb12 (10 μg/ml). However, at the same time point, cells remained negative of viral DNA, as measured by quantitative PCR (qPCR) (Fig. 1b, black bars), indicating that antigen detected in $CD4^+$ T cells was not the product of virus replication in the target cells, but was transmitted from the infected MOLT cells. When HIV antigen-positive target cells were sorted and left for 5 days in the presence of the inhibitors, only the untreated cells remained positive for p24 antigen staining (Fig. 1a, white bars). Proviral DNA detection (Fig. 1b, white bars) and p24 antigen production in the supernatant (Fig. 1c) were only detected in untreated cells, indicating that the antiretrovirals used effectively block virus replication after cell-to-cell transmission.

In lymphoid MT-4 cells, captured virus could be detected as early as 2 h post-coculture, reached a maximum at 24 h, and was maintained for up to 48 h (Fig. 2a). Early flow cytometry detection of intracellular virus antigen may indicate that HIV antigen in short-term cocultures does not accurately measure HIV infectivity. To confirm this hypothesis, total viral DNA in target cells was measured by qPCR. Figure 2b shows that despite massive intracellular p24-antigen detection, TDF and AZT clearly blocked infection even after 48 h post-coculture.

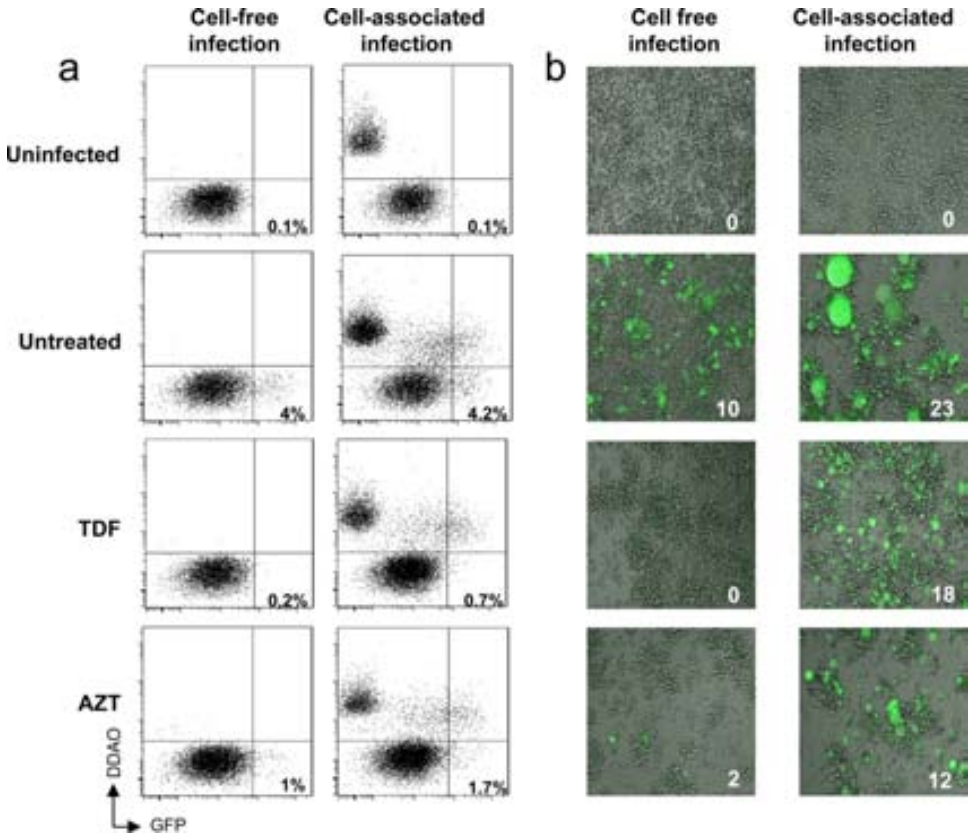


FIG 3 LTR-driven GFP did not accurately measure cell-associated infection or drug susceptibility. CEM-GFP cells were cocultured with uninfected or HIV-1_{NL4-3}-infected MOLT cells or infected by cell-free virus (HIV-1_{NL4-3}) in the presence or absence of 4 μM TDF and 4 μM AZT. Infection of target cells was determined by the percentage of cells positive for GFP signal 48 h postcoculture. (a) Dot plots of flow cytometry analysis corresponding to one representative experiment are shown. In the cell-associated infection assay, double-positive cells were excluded from the analysis for being considered cell-cell fusions, as suggested in reference 36. (b) Total LTR-driven GFP expression in the corresponding cell cultures. Values represent the average fluorescence intensity of the cell culture, as measured by green/gray pixel intensity in GFP fluorescence. Images showing the GFP fluorescence and phase-contrast overlays and the average fluorescence intensity values correspond to one representative experiment of three.

Cell-free and cell-associated HIV infections were equally sensitive to inhibition by reverse transcriptase inhibitors. To compare drug efficacies in cell-free and cell-associated virus transmission, CEM-GFP cells were cocultured with HIV-1_{NL4-3}-infected MOLT cells labeled with DDAO cell tracer or infected with cell-free virus (HIV-1_{NL4-3}), in the presence of various concentrations of the RT inhibitors AZT and TDF. Forty-eight hours postcoculture, infection of target cells was determined by the percentage of cells positive for GFP signal and by proviral DNA detection (Fig. 3 and 4). As indicated in reference 36, care was taken to normalize virus input in both cell-free and cell-associated cultures, leading to roughly identical results in the percentage of GFP^+ cells in the untreated condition at 48 h (Fig. 3a). A significantly high virus concentration, roughly 5 μg/ml of p24 antigen and >25-fold higher of the commonly used virus input in drug susceptibility studies in MT-4 cells (20, 21), was required to achieve 4 to 5% GFP^+ cells in cell-free infections. Under these conditions, both AZT and TDF effectively blocked virus replication with similar 50% effective concentrations (EC_{50}s) measured either by GFP signal or total viral DNA in target cells (Table 1).

Flow cytometry evaluation of LTR-dependent GFP expression in cell-associated infections underestimates virus replication and may not accurately evaluate antiviral efficacy. Cell-to-cell culture of infected and uninfected cells may lead to Tat-dependent transactivation of the HIV LTR in the absence of virus replication (10, 19, 38). Cocultures of HeLa Env^+ cells, expressing Tat and Rev proteins, with CEM-GFP cells were performed to show HIV-1 replication-independent LTR-driven expression of GFP. Transactivation of HIV-1 LTR occurred in the absence of virus replication and could not be inhibited by TDF (see Fig. S1 in the supplemental material).

When comparing cell-free and cell-associated infections under conditions in which GFP expression was normalized to that of target cells by flow cytometry, the average fluorescence intensity (mean fluorescent intensity [MFI]) of the complete culture measured by fluorescence microscopy was higher in cell-associated virus than that in cell-free infections (Fig. 3b). AZT and TDF blocked total GFP expression measured by microscopy in cell-free infections, but inhibition was only partial in cell-associated infections (48% and 25% for AZT and TDF, respectively), indicating that GFP at 48 h was a reflection of both Tat-dependent transactivation and a minor component of effective viral replication. Conversely, flow cytometry data suggest a very similar inhibition in cell-free and cell-associated infections (75% versus 60% for AZT and 95% versus 84% for TDF, comparing cell-free versus cell-associated infections). This is due to the incapacity of flow

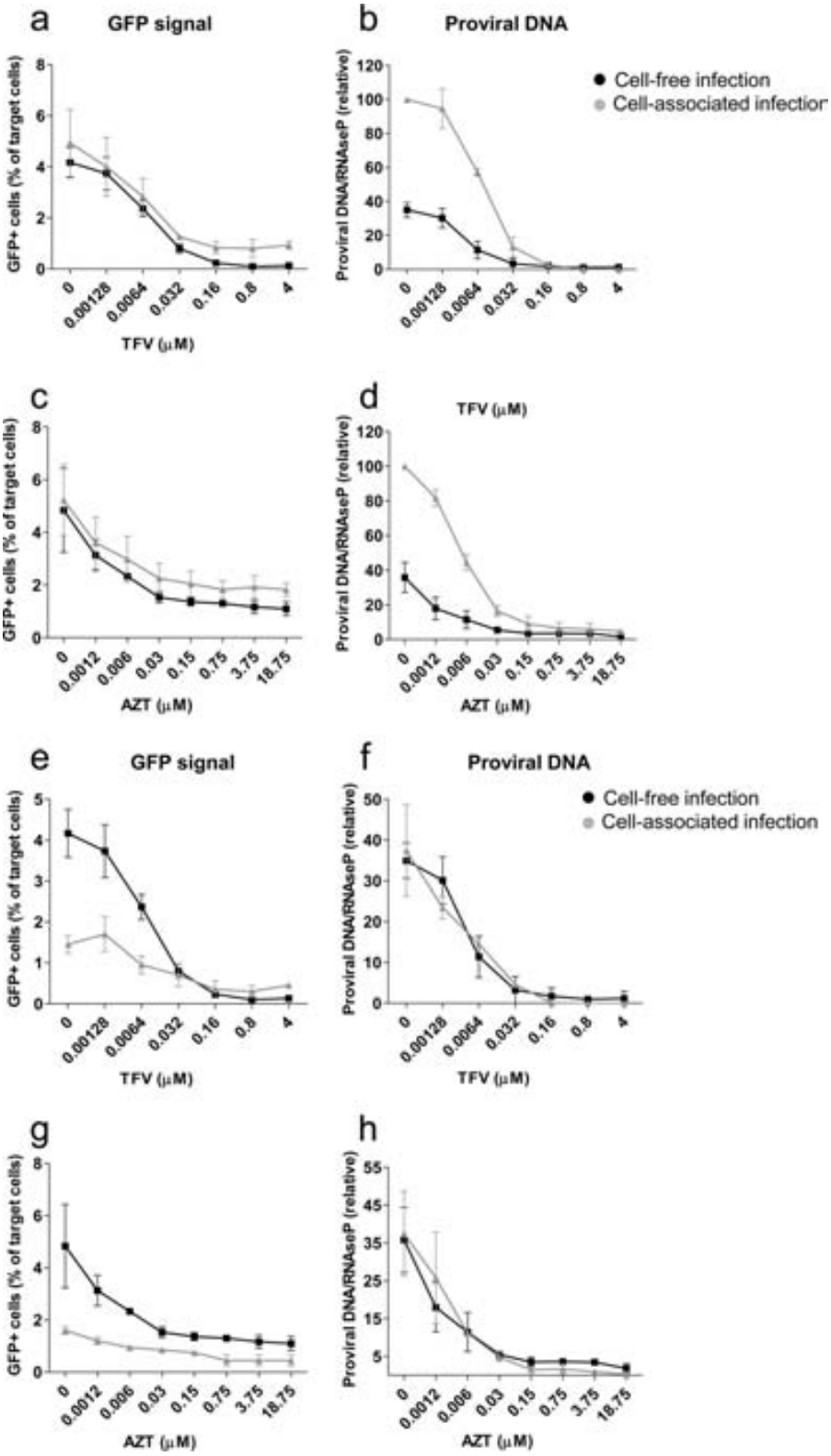


FIG 4 Potent inhibition of HIV replication in cell-free and cell-associated infection as measured by total viral DNA. CEM-GFP cells were either infected with cell-free HIV-1_{NL4-3} or cocultured with HIV-1_{NL4-3}-infected MOLT cells in the presence or the absence of serial dilutions of tenofovir (TDF) and AZT. Forty-eight hours post-coculture, infection of target cells was determined by the percentage of cells positive for GFP signal (left panels), as assessed by flow cytometry, or total viral DNA (right panels) amplified by qPCR and normalized by the cellular housekeeping gene coding for RNAse P. Cell-free (black lines) and cell-associated (gray lines) infections were normalized by the fraction of cells infected in the absence of drug using similar GFP^+ values (a, b, c, and d) or similar proviral DNA copy number (e, f, g, and h). Proviral DNA data are expressed as relative to the untreated condition. Data represent the means ± SD from three independent experiments.

TABLE 1 Similar anti-HIV activities of RTI in cell-free and cell-associated HIV-1 infections

RTI	EC_{50} (μM)[a] of:			
	Cell-free virus		Cell-associated virus	
	GFP	Proviral DNA	GFP	Proviral DNA
TDF	0.0085 ± 0.0012	0.0059 ± 0.001	0.01 ± 0.0039	0.0069 ± 0.0026
AZT	0.0086 ± 0.0054	0.0033 ± 0.0028	0.0122 ± 0.0055	0.0046 ± 0.0007

[a] The 50% effective concentration (EC_{50}) was determined by GFP and proviral DNA measurements after cell-free and cell-associated HIV_{NL4-3} infections in CEM-GFP cells cultured for 48 h to determine the dose-response curves of AZT and TDF. Considering any of the two systems employed to quantify infection, there was no significant difference in the EC_{50}s between the cell-free and cell-to-cell infections. The values shown represent the means ± standard deviations from three independent experiments.

cytometry to quantify GFP^+ giant cell fusions, which on the other hand, may have an important component of Tat-dependent transactivation due to Env-mediated cell fusion.

To further confirm the effect of antiretrovirals in cell-to-cell infections, total viral DNA was measured under conditions in which GFP expression was normalized to that of target cells by flow cytometry (Fig. 4a and c). Total viral DNA was significantly higher in cell-associated infection; however, both AZT and TDF potently blocked total viral DNA with similar potencies (Fig. 4b and d; Table 1). These results could be interpreted as cell-to-cell transmission being a more efficient mechanism of infection. However, when infectivity was normalized to total DNA by reducing the number of infected cells in cocultures, the effects of both AZT and TDF also showed similar potencies (Fig. 4e to h), indicating that total viral DNA is a reflection of the higher multiplicity of infection in cocultures that is not adequately quantified by gating GFP^+ single cells, as indicated by Sigal et al. (36). The lack of total inhibition of GFP^+ cells by AZT in cell-free virus infections may be a reflection of the high virus input that was required to achieve an effect comparable to that observed in cell-associated infection experiments.

The transmission index differs depending on how HIV infection is measured. The transmission index (T_x) represents the ratio between the fraction of infected cells in the presence of drug (I_d) divided by the fraction of cells infected in the absence of drug (I), and it is dependent on the MOI (m), defined as the product of the virus particle number and the infectivity per virus particle (36). The calculated T_xs considering the intracellular p24 antigen staining measurements (i.e., transfer of virus) or the effective HIV infection (i.e., total viral DNA quantification) are completely different (Fig. 5a and b). No inhibition of intracellular virus antigen was noted even in the presence of TDF (4 μM), but complete inhibition of replication 48 h post-coculture was observed (Fig. 3 to 5). According to GFP expression, the potency of TDF or AZT in cell-free virus infection could not be recapitulated in cell-to-cell transmission but was clearly accomplished when total viral DNA was measured (Fig. 5c and d). Thus, AZT and TDF blocked virus replication in cells that were apparently infected, as measured by FACS analysis.

DISCUSSION

Cell-to-cell HIV transmission has often been associated with a more efficient mechanism of infection (16, 17, 32, 34, 37). Infected cells bind and interact with target $CD4^+$ T cells in manners that promote virus transmission through the so-called "virological synapse" in which the interaction between CD4 and the HIV envelope glycoprotein plays a prominent role (22, 31, 35). Effective transmission of virus is a CD4-dependent process leading to virus internalization and uncoating, reverse transcription, and subsequent steps leading to virus production from the infected cell.

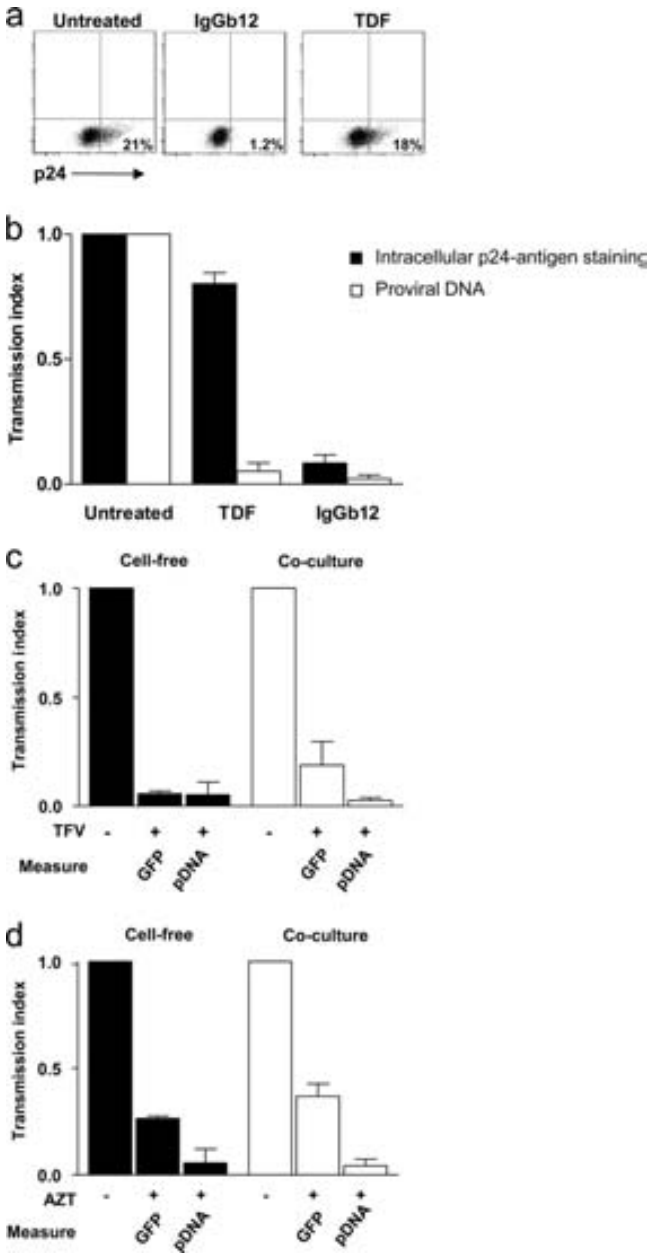


FIG 5 Transmission index (T_x) for cell-free and cell-associated infections. (a) Dot plots of a representative coculture between $HIV\text{-}1_{NL4-3}$-infected MOLT cells and primary $CD4^+$ T cells. Values indicate the percentages of intracellular p24 antigen-positive target cells in each quadrant. (b) Coculture between $HIV\text{-}1_{NL4-3}$-infected MOLT cells and primary $CD4^+$ T lymphocytes was performed for 48 h in the presence or absence of TDF (4 μM) and IgGb12 (10 μg/ml). Data obtained by intracellular p24 antigen staining (black bars) and proviral DNA quantification (white bars) were used to calculate the T_x (36). The T_x was also calculated using GFP data and proviral DNA quantification after cell-free (black bars) and cell-associated (white bars) infections performed for 48 h using CEM-GFP target cells in the presence (+) or absence (−) of 4 μM TDF (TFV) (c) or 4 μM AZT (d). The means ± SD from three independent experiments are shown.

Cell-to-cell transmission was recently shown as a mechanism to evade inhibition only by the anti-gp120 neutralizing antibodies, including CD4-IgG2 (Pro542), but not when other entry inhibitors were used, such as gp41-directed or cell-directed antibodies (1). To this end, virus strains requiring addition of DEAE-dextran for cell-free but not for cell-to-cell infection were used in nonlymphoid (HeLa TZM-bl) cells. DEAE-dextran is a nonspecific polycation commonly used to enhance the association of viruses, including HIV, with target cells via relatively nonspecific charge interactions (2, 23) and may severely affect gp120 interactions with the cell surface such as to interfere with the inhibitory effect of gp120-targeting neutralizing antibodies. The results in reference 1 are in contrast to previous observations that virus attachment inhibitors, including CD4-IgG2 (7) and others (24) effectively block cell-to-cell transmission with equal potency to cell-free transmission.

We and others have shown that cell-to-cell contact may lead to the transfer of HIV particles through an endocytic internalization of fully coated virions, readily detectable in $CD4^+$ target cells (8, 22). However, endocytosis as a means to productive infection is a matter of ample debate. Early and recent evidence indicates that HIV may fuse with endosomal membranes to initiate a productive infection (13, 15, 28, 30), questioning the general understanding that virus entry occurs at the cell surface. Here, we recapitulate these results to demonstrate that evaluation of the presence of virus antigen in target cells at early time points does not accurately measure infectivity or drug efficacy. Conversely, detection of total viral DNA (proviral DNA) as measured by qPCR clearly demonstrates that cells remained infection negative in the presence of antiretroviral agents. Our results contrast with those of Sigal et al. (36), who evaluated intracellular antigen in cocultures of infected and uninfected cells and assumed that the presence of intracellular HIV antigen in short-term cocultures accurately measured HIV infectivity. Furthermore, to compare cell-free and cell-associated viral transmission, we used a method based on measurement of LTR-driven expression of a given marker to evaluate virus replication in cell-free virus infections, similar to that used by Sigal et al. (36). A similar multiplicity of infection (*m*), defined as the number of GFP-positive target cells in the absence of drug, was used to compare drug efficacies in both infection systems. In line with previous observations showing that both cell-to-cell spread and cell-free viral spread were equally sensitive to entry inhibition (7, 24), drug response curves demonstrated equal efficacy of antiretrovirals, strongly indicating that cell-to-cell transmission may not allow for ongoing virus replication in the presence of antiretroviral therapy.

Similarly to Sigal et al. we have found that antiretroviral drugs differentially blocked GFP expression, which may lead to the assumption that antiretroviral agents effectively block cell-free virus but fail to completely inhibit HIV-1 cell-to-cell spread. However, we show complete inhibition of proviral DNA in the same target cells, which, in contrast with the first assumption, points to a similar efficiency of antiretrovirals in both modes of transmission.

As previously shown (10, 19), and unlike cell-free virus infection, AZT or TDF cannot block cell-to-cell-mediated Tat-dependent transactivation in the absence of replication. Evaluation of cell cultures by fluorescence microscopy demonstrated that this effect could have important consequences in cell-associated infection when syncytium formation is not impaired by the antiretroviral agent tested. Moreover, the flow cytometry analysis does not entirely evaluate infection as giant syncytia cannot be analyzed. Under these conditions, the potency of antiretroviral agents is being underestimated. Thus, GFP expression may not accurately evaluate drug efficacy in cell-associated virus transmission.

The value of *m* is similar to the well-known definition of multiplicity of infection (MOI), that is, the ratio of infectious agents per target cell, a concept commonly used to normalize virus titers prior to the evaluation of drug efficacy or virus drug resistance in cell culture experiments. The convention, when comparing the efficacy of a drug against two independent virus strains (or two mechanisms of virus transfer), is to normalize the virus input and then determine the efficacy of the drug, which is commonly reported as the fold change (FC) in EC_{50}s. Assuming a similar MOI, the probability to propagate two infectious viruses (or two mechanisms of infection of the same virus) is the same, i.e., the infectivity of a virus particle does not increase by increasing the number of particles (with a higher MOI). A higher MOI raises the number of infectious events. Screening of anti-HIV agents is commonly tested at a low MOI, and plasma viral load is a predictor of treatment efficacy (26). Sigal et al. conclude that infectivity through cell-to-cell transfer is resistant to the drug, but they have failed to evaluate both conditions (cell-free versus cell-associated infection) at similar MOI, by incorrectly assuming that each virus transferred in coculture will lead to a productive infection.

We concur with the hypothesis that multiple factors affect HIV persistence in the presence of antiretroviral treatment. However, assuming that cell-to-cell spread is permitting ongoing replication despite antiretroviral therapy is difficult to ascertain; although cell-to-cell spread has been well documented *in vitro*, its relevance in patients is still to be defined. How virus reservoirs are maintained in the presence of therapy has important clinical implications and might be the main barrier to complete clearance of HIV (33). Therefore, data on cell-to-cell spread should be taken with caution as it is crucial to correctly distinguish and measure abortive virus transfer or surrogate markers of infection (LTR-driven GFP) from effective viral replication.

ACKNOWLEDGMENTS

Cells, drugs, and viruses were received from the EU Programme EVA Centralised Facility for AIDS Reagents, NIBSC, United Kingdom (AVIP contract no. LSHP-CT-2004-503487) or the National Institutes of Health (AIDS Research and Reference Reagent Program).

This work was supported in part by the Spanish Ministerio de Economia y Consumo (BFU2009-06958 and SAF2007-63622-02), FIPSE 360783-09 (J.A.E.), and Gala contra la SIDA 2011.

M.P. and E.B. are fellows of the Spanish Fondo de Investigación Sanitaria (FIS).

REFERENCES

1. **Abela IA, et al.** 2012. Cell-cell transmission enables HIV-1 to evade inhibition by potent CD4bs directed antibodies. PLoS Pathog. **8**:e1002634. doi:10.1371/journal.ppat.1002634.
2. **Ahn KS, Ou W, Silver J.** 2004. Inhibition of certain strains of HIV-1 by cell surface polyanions in the form of cholesterol-labeled oligonucleotides. Virology **330**:50–61.
3. **Ballana E, et al.** 2009. Cell adhesion through alphaV-containing integrins is required for efficient HIV-1 infection in macrophages. Blood **113**:1278–1286.
4. **Ballana E, et al.** 2010. ZNRD1 (zinc ribbon domain-containing 1) is a host cellular factor that influences HIV-1 replication and disease progression. Clin. Infect. Dis. **50**:1022–1032.
5. **Blanco J, Barretina J, Clotet B, Este JA.** 2004. R5 HIV gp120-mediated cellular contacts induce the death of single CCR5-expressing CD4 T cells by a gp41-dependent mechanism. J. Leukoc. Biol. **76**:804–811.

6. **Blanco J, et al.** 2004. High level of coreceptor-independent HIV transfer induced by contacts between primary CD4 T cells. J. Biol. Chem. **279**: 51305–51314.
7. **Bosch B, et al.** 2005. Inhibition of coreceptor independent cell-to-cell HIV-1 transmission by a CD4-IgG2 fusion protein. Antimicrob. Agents Chemother. **49**:4296–4304.
8. **Bosch B, et al.** 2008. A clathrin-dynamin-dependent endocytic pathway for the uptake of HIV-1 by direct T cell-T cell transmission. Antiviral Res. **80**:185–193.
9. **Chen P, Hubner W, Spinelli MA, Chen BK.** 2007. Predominant mode of human immunodeficiency virus transfer between T cells is mediated by sustained Env-dependent neutralization-resistant virological synapses. J. Virol. **81**:12582–12595.
10. **Clavel F, Charneau P.** 1994. Fusion from without directed by human immunodeficiency virus particles. J. Virol. **68**:1179–1185.
11. **Clotet-Codina I, et al.** 2009. HIV endocytosis after dendritic cell to T cell viral transfer leads to productive virus infection. Antiviral Res. **83**:94–98.
12. **Dahl, V, Josefsson L, Palmer S.** 2010. HIV reservoirs, latency, and reactivation: prospects for eradication. Antiviral Res. **85**:286–294.
13. **Dale BM, et al.** 2011. Cell-to-cell transfer of HIV-1 via virological synapses leads to endosomal virion maturation that activates viral membrane fusion. Cell Host Microbe **10**:551–562.
14. **Deeks SG.** 2011. HIV: how to escape treatment. Nature **477**:36–37.
15. **de la Vega M, et al.** 2011. Inhibition of HIV-1 endocytosis allows lipid mixing at the plasma membrane, but not complete fusion. Retrovirology **8**:99. doi:10.1186/1742-4690-8-99.
16. **Del Portillo A, et al.** 2011. Multiploid inheritance of HIV-1 during cell-to-cell infection. J. Virol. **85**:7169–7176.
17. **Dimitrov DS, et al.** 1993. Quantitation of human immunodeficiency virus type 1 infection kinetics. J. Virol. **67**:2182–2190.
18. **Esté JA, Cihlar T.** 2010. Current challenges in antiretoviral research and treatment. Antiviral Res. **85**:26–33.
19. **Este JA, et al.** 1995. Inhibition of HIV type 1 Tat-mediated transactivation by oncostatin M in HLtat cells. AIDS Res. Hum. Retroviruses **11**:1355–1358.
20. **Gonzalez E, Ballana E, Clotet B, Este JA.** 2011. Development of resistance to VIR-353 with cross-resistance to the natural HIV-1 entry virus inhibitory peptide (VIRIP). AIDS **25**:1557–1583.
21. **Gonzalez-Ortega E, et al.** 2010. ADS-J1 inhibits HIV-1 entry by interacting with gp120 and does not block the fusion-active gp41 core formation. Antimicrob. Agents Chemother. **54**:4487–4492.
22. **Hubner W, et al.** 2009. Quantitative 3D video microscopy of HIV transfer across T cell virological synapses. Science **323**:1743–1747.
23. **Konopka K, Stamatatos L, Larsen CE, Davis BR, Duzgunes N.** 1991. Enhancement of human immunodeficiency virus type 1 infection by cationic liposomes: the role of CD4, serum and liposome-cell interactions. J. Gen. Virol. **72**:2685–2696.
24. **Martin N, et al.** 2010. Virological synapse-mediated spread of human immunodeficiency virus type 1 between T cells is sensitive to entry inhibition. J. Virol. **84**:3516–3527.
25. **Massanella M, et al.** 2009. Antigp41 antibodies fail to block early events of virological synapses but inhibit HIV spread between T cells. AIDS **23**:183–188.
26. **Mellors JW, et al.** 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science **272**:1167–1170.
27. **Mercer J, Schelhaas M, Helenius A.** 2010. Virus entry by endocytosis. Annu. Rev. Biochem. **79**:803–833.
28. **Miyauchi K, Kim Y, Latinovic O, Morozov V, Melikyan GB.** 2009. HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. Cell **137**:433–444.
29. **Moncunill G, et al.** 2008. Anti-HIV activity and resistance profile of the CXC chemokine receptor 4 antagonist POL3026. Mol. Pharmacol. **73**: 1264–1273.
30. **Pauza CD, Price TM.** 1988. Human immunodeficiency virus infection of T cells and monocytes proceeds via receptor-mediated endocytosis. J. Cell Biol. **107**:959–968.
31. **Permanyer M, Ballana E, Este JA.** 2010. Endocytosis of HIV: anything goes. Trends Microbiol. **18**:543–551.
32. **Phillips DM.** 1994. The role of cell-to-cell transmission in HIV infection. AIDS **8**:719–731.
33. **Richman DD, et al.** 2009. The challenge of finding a cure for HIV infection. Science **323**:1304–1307.
34. **Sato H, Orenstein J, Dimitrov D, Martin M.** 1992. Cell-to-cell spread of HIV-1 occurs within minutes and may not involve the participation of virus particles. Virology **186**:712–724.
35. **Sattentau Q.** 2008. Avoiding the void: cell-to-cell spread of human viruses. Nat. Rev. Microbiol. **6**:815–826.
36. **Sigal A, et al.** 2011. Cell-to-cell spread of HIV permits ongoing replication despite antiretroviral therapy. Nature **477**:95–98.
37. **Sourisseau M, Sol-Foulon N, Porrot F, Blanchet F, Schwartz O.** 2007. Inefficient human immunodeficiency virus replication in mobile lymphocytes. J. Virol. **81**:1000–1012.
38. **Witvrouw M, et al.** 1997. Cell type-dependent effect of sodium valproate on human immunodeficiency virus type 1 replication in vitro. AIDS Res. Hum. Retroviruses **13**:187–192.

REFERENCES

1. Engelman A, Cherepanov P: **The structural biology of HIV-1: mechanistic and therapeutic insights.** *Nat Rev Microbiol* 2012, **10:**279-290.

2. Miller CJ: **HIV transmission: migratory Langerhans cells are primary targets in vaginal HIV transmission.** *Immunol Cell Biol* 2007, **85:**269-270.

3. Sattentau Q: **Avoiding the void: cell-to-cell spread of human viruses.** *Nat Rev Microbiol* 2008, **6:**815-826.

4. Piguet V, Sattentau Q: **Dangerous liaisons at the virological synapse.** *J Clin Invest* 2004, **114:**605-610.

5. Sigal A, Kim JT, Balazs AB, Dekel E, Mayo A, Milo R, Baltimore D: **Cell-to-cell spread of HIV permits ongoing replication despite antiretroviral therapy.** *Nature* 2011, **477:**95-98.

6. Centers for Disease C: **Pneumocystis pneumonia--Los Angeles.** *MMWR Morb Mortal Wkly Rep* 1981, **30:**250-252.

7. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, et al: **Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).** *Science* 1983, **220:**868-871.

8. Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, Palker TJ, Redfield R, Oleske J, Safai B, et al.: **Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS.** *Science* 1984, **224:**500-503.

9. Levy JA, Hoffman AD, Kramer SM, Landis JA, Shimabukuro JM, Oshiro LS: **Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS.** *Science* 1984, **225:**840-842.

10. Coffin J, Haase A, Levy JA, Montagnier L, Oroszlan S, Teich N, Temin H, Toyoshima K, Varmus H, Vogt P, et al.: **Human immunodeficiency viruses.** *Science* 1986, **232:**697.

11. UNAIDS: **2012 UNAIDS Report on the global AIDS epidemic** *UNAIDS Corporate publications* 2012.

12. Shattock RJ, Moore JP: **Inhibiting sexual transmission of HIV-1 infection.** *Nat Rev Microbiol* 2003, **1:**25-34.

13. Bobardt MD, Chatterji U, Selvarajah S, Van der Schueren B, David G, Kahn B, Gallay PA: **Cell-free human immunodeficiency virus type 1 transcytosis through primary genital epithelial cells.** *J Virol* 2007, **81:**395-405.

14. Hladik F, Sakchalathorn P, Ballweber L, Lentz G, Fialkow M, Eschenbach D, McElrath MJ: **Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1.** *Immunity* 2007, **26:**257-270.

15. Este JA, Telenti A: **HIV entry inhibitors.** *Lancet* 2007, **370:**81-88.

16. Tilton JC, Doms RW: **Entry inhibitors in the treatment of HIV-1 infection.** *Antiviral Res* 2010, **85:**91-100.

17. Haqqani AA, Tilton JC: **Entry inhibitors and their use in the treatment of HIV-1 infection.** *Antiviral Res* 2013, **98:**158-170.

18. Sourisseau M, Sol-Foulon N, Porrot F, Blanchet F, Schwartz O: **Inefficient human immunodeficiency virus replication in mobile lymphocytes.** *J Virol* 2007, **81:**1000-1012.

19. Chen P, Hubner W, Spinelli MA, Chen BK: **Predominant mode of human immunodeficiency virus transfer between T cells is mediated by sustained Env-dependent neutralization-resistant virological synapses.** *J Virol* 2007, **81:**12582-12595.

20. Dimitrov DS, Willey RL, Sato H, Chang LJ, Blumenthal R, Martin MA: **Quantitation of human immunodeficiency virus type 1 infection kinetics.** *J Virol* 1993, **67:**2182-2190.

21. Russell RA, Martin N, Mitar I, Jones E, Sattentau QJ: **Multiple proviral integration events after virological synapse-mediated HIV-1 spread.** *Virology* 2013, **443:**143-149.

22. Del Portillo A, Tripodi J, Najfeld V, Wodarz D, Levy DN, Chen BK: **Multiploid inheritance of HIV-1 during cell-to-cell infection.** *J Virol* 2011, **85:**7169-7176.

23. Igakura T, Stinchcombe JC, Goon PK, Taylor GP, Weber JN, Griffiths GM, Tanaka Y, Osame M, Bangham CR: **Spread of HTLV-I between lymphocytes by virus-induced polarization of the cytoskeleton.** *Science* 2003, **299:**1713-1716.

24. Murooka TT, Deruaz M, Marangoni F, Vrbanac VD, Seung E, von Andrian UH, Tager AM, Luster AD, Mempel TR: **HIV-infected T cells are migratory vehicles for viral dissemination.** *Nature* 2012, **490:**283-287.

25. Singer SJ: **Intercellular communication and cell-cell adhesion.** *Science* 1992, **255:**1671-1677.

26. Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A: **Three-dimensional segregation of supramolecular activation clusters in T cells.** *Nature* 1998, **395:**82-86.

27. Cameron PU, Freudenthal PS, Barker JM, Gezelter S, Inaba K, Steinman RM: **Dendritic cells exposed to human immunodeficiency virus type-1 transmit a vigorous cytopathic infection to CD4+ T cells.** *Science* 1992, **257:**383-387.

28. Pope M, Betjes MG, Romani N, Hirmand H, Cameron PU, Hoffman L, Gezelter S, Schuler G, Steinman RM: **Conjugates of dendritic cells and memory T lymphocytes from skin facilitate productive infection with HIV-1.** *Cell* 1994, **78:**389-398.

29. McDonald D, Wu L, Bohks SM, KewalRamani VN, Unutmaz D, Hope TJ: **Recruitment of HIV and its receptors to dendritic cell-T cell junctions.** *Science* 2003, **300:**1295-1297.

30. Jolly C, Kashefi K, Hollinshead M, Sattentau QJ: **HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse.** *J Exp Med* 2004, **199:**283-293.

31. Arrighi JF, Pion M, Garcia E, Escola JM, van Kooyk Y, Geijtenbeek TB, Piguet V: **DC-SIGN-mediated infectious synapse formation enhances X4 HIV-1 transmission from dendritic cells to T cells.** *J Exp Med* 2004, **200:**1279-1288.

32. Hubner W, McNerney GP, Chen P, Dale BM, Gordon RE, Chuang FY, Li XD, Asmuth DM, Huser T, Chen BK: **Quantitative 3D video microscopy of HIV transfer across T cell virological synapses.** *Science* 2009, **323:**1743-1747.

33. Felts RL, Narayan K, Estes JD, Shi D, Trubey CM, Fu J, Hartnell LM, Ruthel GT, Schneider DK, Nagashima K, et al: **3D visualization of HIV transfer at the virological synapse between dendritic cells and T cells.** *Proc Natl Acad Sci U S A* 2010, **107:**13336-13341.

34. Groot F, Welsch S, Sattentau QJ: **Efficient HIV-1 transmission from macrophages to T cells across transient virological synapses.** *Blood* 2008, **111:**4660-4663.

35. Bosch B, Grigorov B, Senserrich J, Clotet B, Darlix JL, Muriaux D, Este JA: **A clathrin-dynamin-dependent endocytic pathway for the uptake of HIV-1 by direct T cell-T cell transmission.** *Antiviral Res* 2008, **80:**185-193.

36. Blanco J, Bosch B, Fernandez-Figueras MT, Barretina J, Clotet B, Este JA: **High level of coreceptor-independent HIV transfer induced by contacts between primary CD4 T cells.** *J Biol Chem* 2004, **279:**51305-51314.

37. Jolly C, Mitar I, Sattentau QJ: **Requirement for an intact T-cell actin and tubulin cytoskeleton for efficient assembly and spread of human immunodeficiency virus type 1.** *J Virol* 2007, **81:**5547-5560.

38. Jolly C, Mitar I, Sattentau QJ: **Adhesion molecule interactions facilitate human immunodeficiency virus type 1-induced virological synapse formation between T cells.** *J Virol* 2007, **81:**13916-13921.

39. Puigdomenech I, Massanella M, Izquierdo-Useros N, Ruiz-Hernandez R, Curriu M, Bofill M, Martinez-Picado J, Juan M, Clotet B, Blanco J: **HIV transfer between CD4 T cells does not require LFA-1 binding to ICAM-1 and is governed by the interaction of HIV envelope glycoprotein with CD4.** *Retrovirology* 2008, **5:**32.

40. Jolly C, Welsch S, Michor S, Sattentau QJ: **The regulated secretory pathway in CD4(+) T cells contributes to human immunodeficiency virus type-1 cell-to-cell spread at the virological synapse.** *PLoS Pathog* 2011, **7:**e1002226.

41. Jolly C, Sattentau QJ: **Human immunodeficiency virus type 1 virological synapse formation in T cells requires lipid raft integrity.** *J Virol* 2005, **79:**12088-12094.

42. Sol-Foulon N, Sourisseau M, Porrot F, Thoulouze MI, Trouillet C, Nobile C, Blanchet F, di Bartolo V, Noraz N, Taylor N, et al: **ZAP-70 kinase regulates HIV cell-to-cell spread and virological synapse formation.** *EMBO J* 2007, **26:**516-526.

43. Alfsen A, Yu H, Magerus-Chatinet A, Schmitt A, Bomsel M: **HIV-1-infected blood mononuclear cells form an integrin- and agrin-dependent viral synapse to induce efficient HIV-1 transcytosis across epithelial cell monolayer.** *Mol Biol Cell* 2005, **16:**4267-4279.

44. Bomsel M: **Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier.** *Nat Med* 1997, **3:**42-47.

45. Abela IA, Berlinger L, Schanz M, Reynell L, Gunthard HF, Rusert P, Trkola A: **Cell-cell transmission enables HIV-1 to evade inhibition by potent CD4bs directed antibodies.** *PLoS Pathog* 2012, **8:**e1002634.

46. Dale BM, McNerney GP, Thompson DL, Hubner W, de Los Reyes K, Chuang FY, Huser T, Chen BK: **Cell-to-cell transfer of HIV-1 via virological synapses leads to endosomal virion maturation that activates viral membrane fusion.** *Cell Host Microbe* 2011, **10:**551-562.

47. Favoreel HW, Van Minnebruggen G, Van de Walle GR, Ficinska J, Nauwynck HJ: **Herpesvirus interference with virus-specific antibodies: bridging antibodies, internalizing antibodies, and hiding from antibodies.** *Vet Microbiol* 2006, **113:**257-263.

48. Law M, Hollinshead R, Smith GL: **Antibody-sensitive and antibody-resistant cell-to-cell spread by vaccinia virus: role of the A33R protein in antibody-resistant spread.** *J Gen Virol* 2002, **83:**209-222.

49. Timpe JM, Stamataki Z, Jennings A, Hu K, Farquhar MJ, Harris HJ, Schwarz A, Desombere I, Roels GL, Balfe P, McKeating JA: **Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies.** *Hepatology* 2008, **47:**17-24.

50. Gupta P, Balachandran R, Ho M, Enrico A, Rinaldo C: **Cell-to-cell transmission of human immunodeficiency virus type 1 in the presence of azidothymidine and neutralizing antibody.** *J Virol* 1989, **63:**2361-2365.

51. Bosch B, Blanco J, Pauls E, Clotet-Codina I, Armand-Ugon M, Grigorov B, Muriaux D, Clotet B, Darlix JL, Este JA: **Inhibition of coreceptor-independent cell-to-cell human immunodeficiency virus type 1 transmission by a CD4-immunoglobulin G2 fusion protein.** *Antimicrob Agents Chemother* 2005, **49:**4296-4304.

52. Massanella M, Puigdomenech I, Cabrera C, Fernandez-Figueras MT, Aucher A, Gaibelet G, Hudrisier D, Garcia E, Bofill M, Clotet B, Blanco J: **Antigp41 antibodies fail to block early events of virological synapses but inhibit HIV spread between T cells.** *AIDS* 2009, **23:**183-188.

53. Martin N, Welsch S, Jolly C, Briggs JA, Vaux D, Sattentau QJ: **Virological synapse-mediated spread of human immunodeficiency virus type 1 between T cells is sensitive to entry inhibition.** *J Virol* 2010, **84:**3516-3527.

54. Miyauchi K, Kim Y, Latinovic O, Morozov V, Melikyan GB: **HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes.** *Cell* 2009, **137:**433-444.

55. Yu D, Wang W, Yoder A, Spear M, Wu Y: **The HIV envelope but not VSV glycoprotein is capable of mediating HIV latent infection of resting CD4 T cells.** *PLoS Pathog* 2009, **5:**e1000633.

56. Wu L, KewalRamani VN: **Dendritic-cell interactions with HIV: infection and viral dissemination.** *Nat Rev Immunol* 2006, **6:**859-868.

57. Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, Middel J, Cornelissen IL, Nottet HS, KewalRamani VN, Littman DR, et al: **DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells.** *Cell* 2000, **100:**587-597.

58. van Kooyk Y, Geijtenbeek TB: **DC-SIGN: escape mechanism for pathogens.** *Nat Rev Immunol* 2003, **3:**697-709.

59. Boggiano C, Manel N, Littman DR: **Dendritic cell-mediated trans-enhancement of human immunodeficiency virus type 1 infectivity is independent of DC-SIGN.** *J Virol* 2007, **81:**2519-2523.

60. Baribaud F, Pohlmann S, Leslie G, Mortari F, Doms RW: **Quantitative expression and virus transmission analysis of DC-SIGN on monocyte-derived dendritic cells.** *J Virol* 2002, **76:**9135-9142.

61. Gummuluru S, Rogel M, Stamatatos L, Emerman M: **Binding of human immunodeficiency virus type 1 to immature dendritic cells can occur independently of DC-SIGN and mannose binding C-type lectin receptors via a cholesterol-dependent pathway.** *J Virol* 2003, **77:**12865-12874.

62. Izquierdo-Useros N, Lorizate M, Contreras FX, Rodriguez-Plata MT, Glass B, Erkizia I, Prado JG, Casas J, Fabrias G, Krausslich HG, Martinez-Picado J: **Sialyllactose in viral membrane gangliosides is a novel molecular recognition pattern for mature dendritic cell capture of HIV-1.** *PLoS Biol* 2012, **10:**e1001315.

63. Izquierdo-Useros N, Lorizate M, Puertas MC, Rodriguez-Plata MT, Zangger N, Erikson E, Pino M, Erkizia I, Glass B, Clotet B, et al: **Siglec-1 is a novel dendritic cell receptor that mediates HIV-1 trans-infection through recognition of viral membrane gangliosides.** *PLoS Biol* 2012, **10:**e1001448.

64. Puryear WB, Yu X, Ramirez NP, Reinhard BM, Gummuluru S: **HIV-1 incorporation of host-cell-derived glycosphingolipid GM3 allows for capture by mature dendritic cells.** *Proc Natl Acad Sci U S A* 2012, **109:**7475-7480.

65. Garcia E, Pion M, Pelchen-Matthews A, Collinson L, Arrighi JF, Blot G, Leuba F, Escola JM, Demaurex N, Marsh M, Piguet V: **HIV-1 trafficking to the dendritic cell-T-cell infectious synapse uses a pathway of tetraspanin sorting to the immunological synapse.** *Traffic* 2005, **6:**488-501.

66. Alfsen A, Iniguez P, Bouguyon E, Bomsel M: **Secretory IgA specific for a conserved epitope on gp41 envelope glycoprotein inhibits epithelial transcytosis of HIV-1.** *J Immunol* 2001, **166:**6257-6265.

67. Sherer NM, Lehmann MJ, Jimenez-Soto LF, Horensavitz C, Pypaert M, Mothes W: **Retroviruses can establish filopodial bridges for efficient cell-to-cell transmission.** *Nat Cell Biol* 2007, **9:**310-315.

68. Sowinski S, Jolly C, Berninghausen O, Purbhoo MA, Chauveau A, Kohler K, Oddos S, Eissmann P, Brodsky FM, Hopkins C, et al: **Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission.** *Nat Cell Biol* 2008, **10:**211-219.

69. Berard M, Tough DF: **Qualitative differences between naive and memory T cells.** *Immunology* 2002, **106:**127-138.

70. Iezzi G, Karjalainen K, Lanzavecchia A: **The duration of antigenic stimulation determines the fate of naive and effector T cells.** *Immunity* 1998, **8:**89-95.

71. Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, Boucher G, Boulassel MR, Ghattas G, Brenchley JM, et al: **HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation.** *Nat Med* 2009, **15:**893-900.

72. Schnittman SM, Lane HC, Greenhouse J, Justement JS, Baseler M, Fauci AS: **Preferential infection of CD4+ memory T cells by human immunodeficiency virus type 1: evidence for a role in the selective T-cell functional defects observed in infected individuals.** *Proc Natl Acad Sci U S A* 1990, **87:**6058-6062.

73. Helbert MR, Walter J, L'Age J, Beverley PC: **HIV infection of CD45RA+ and CD45RO+ CD4+ T cells.** *Clin Exp Immunol* 1997, **107:**300-305.

74. Roederer M, Raju PA, Mitra DK, Herzenberg LA, Herzenberg LA: **HIV does not replicate in naive CD4 T cells stimulated with CD3/CD28.** *J Clin Invest* 1997, **99:**1555-1564.

75. Spina CA, Prince HE, Richman DD: **Preferential replication of HIV-1 in the CD45RO memory cell subset of primary CD4 lymphocytes in vitro.** *J Clin Invest* 1997, **99:**1774-1785.

76. Dai J, Agosto LM, Baytop C, Yu JJ, Pace MJ, Liszewski MK, O'Doherty U: **Human immunodeficiency virus integrates directly into naive resting CD4+ T cells but enters naive cells less efficiently than memory cells.** *J Virol* 2009, **83:**4528-4537.

77. Wang W, Guo J, Yu D, Vorster PJ, Chen W, Wu Y: **A dichotomy in cortical actin and chemotactic actin activity between human memory and naive T cells contributes to their differential susceptibility to HIV-1 infection.** *J Biol Chem* 2012, **287:**35455-35469.

78. Tardif MR, Tremblay MJ: **LFA-1 is a key determinant for preferential infection of memory CD4+ T cells by human immunodeficiency virus type 1.** *J Virol* 2005, **79:**13714-13724.

79. Adamson CS, Freed EO: **Novel approaches to inhibiting HIV-1 replication.** *Antiviral Res* 2010, **85:**119-141.

80. Marsh M, Helenius A: **Virus entry: open sesame.** *Cell* 2006, **124:**729-740.

81. Izquierdo-Useros N, Esteban O, Rodriguez-Plata MT, Erkizia I, Prado JG, Blanco J, Garcia-Parajo MF, Martinez-Picado J: **Dynamic imaging of cell-free and cell-associated viral capture in mature dendritic cells.** *Traffic* 2011, **12:**1702-1713.

82. Permanyer M, Ballana E, Este JA: **Endocytosis of HIV: anything goes.** *Trends Microbiol* 2010, **18:**543-551.

83. Jolly C, Sattentau QJ: **Retroviral spread by induction of virological synapses.** *Traffic* 2004, **5:**643-650.

84. Stein BS, Gowda SD, Lifson JD, Penhallow RC, Bensch KG, Engleman EG: **pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane.** *Cell* 1987, **49:**659-668.

85. Bedinger P, Moriarty A, von Borstel RC, 2nd, Donovan NJ, Steimer KS, Littman DR: **Internalization of the human immunodeficiency virus does not require the cytoplasmic domain of CD4.** *Nature* 1988, **334:**162-165.

86. Kielian M, Jungerwirth S: **Mechanisms of enveloped virus entry into cells.** *Mol Biol Med* 1990, **7:**17-31.

87. Skehel JJ, Wiley DC: **Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin.** *Annu Rev Biochem* 2000, **69:**531-569.

88. Clotet-Codina I, Bosch B, Senserrich J, Fernandez-Figueras MT, Pena R, Ballana E, Bofill M, Clotet B, Este JA: **HIV endocytosis after dendritic cell to T cell viral transfer leads to productive virus infection.** *Antiviral Res* 2009, **83:**94-98.

89. Pauza CD, Price TM: **Human immunodeficiency virus infection of T cells and monocytes proceeds via receptor-mediated endocytosis.** *J Cell Biol* 1988, **107:**959-968.

90. Daecke J, Fackler OT, Dittmar MT, Krausslich HG: **Involvement of clathrin-mediated endocytosis in human immunodeficiency virus type 1 entry.** *J Virol* 2005, **79:**1581-1594.

91. de la Vega M, Marin M, Kondo N, Miyauchi K, Kim Y, Epand RF, Epand RM, Melikyan GB: **Inhibition of HIV-1 endocytosis allows lipid mixing at the plasma membrane, but not complete fusion.** *Retrovirology* 2011, **8:**99.

92. Sattentau QJ: **Cell-to-Cell Spread of Retroviruses.** *Viruses* 2010, **2:**1306-1321.

93. Ballana E, Pauls E, Clotet B, Perron-Sierra F, Tucker GC, Este JA: **beta5 integrin is the major contributor to the alphaVintegrin-mediated blockade of HIV-1 replication.** *J Immunol* 2011, **186:**464-470.

94. Ballana E, Pauls E, Senserrich J, Clotet B, Perron-Sierra F, Tucker GC, Este JA: **Cell adhesion through alphaV-containing integrins is required for efficient HIV-1 infection in macrophages.** *Blood* 2009, **113:**1278-1286.

95. Ballana E, Senserrich J, Pauls E, Faner R, Mercader JM, Uyttebroeck F, Palou E, Mena MP, Grau E, Clotet B, et al: **ZNRD1 (zinc ribbon domain-containing 1) is a host cellular factor that influences HIV-1 replication and disease progression.** *Clin Infect Dis* 2010, **50:**1022-1032.

96. Cavrois M, De Noronha C, Greene WC: **A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes.** *Nat Biotechnol* 2002, **20:**1151-1154.

97. Doitsh G, Cavrois M, Lassen KG, Zepeda O, Yang Z, Santiago ML, Hebbeler AM, Greene WC: **Abortive HIV infection mediates CD4 T cell depletion and inflammation in human lymphoid tissue.** *Cell* 2010, **143:**789-801.

98. Yoder A, Yu D, Dong L, Iyer SR, Xu X, Kelly J, Liu J, Wang W, Vorster PJ, Agulto L, et al: **HIV envelope-CXCR4 signaling activates cofilin to overcome cortical actin restriction in resting CD4 T cells.** *Cell* 2008, **134:**782-792.

99. Pelchen-Matthews A, Parsons IJ, Marsh M: **Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from p56lck, increased association with clathrin-coated pits, and altered endosomal sorting.** *J Exp Med* 1993, **178:**1209-1222.

100. Permanyer M, Ballana E, Ruiz A, Badia R, Riveira-Munoz E, Gonzalo E, Clotet B, Este JA: **Antiretroviral agents effectively block HIV replication after cell-to-cell transfer.** *J Virol* 2012, **86:**8773-8780.

101. Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T: **Dynasore, a cell-permeable inhibitor of dynamin.** *Dev Cell* 2006, **10:**839-850.

102. von Kleist L, Stahlschmidt W, Bulut H, Gromova K, Puchkov D, Robertson MJ, MacGregor KA, Tomilin N, Pechstein A, Chau N, et al: **Role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition.** *Cell* 2011, **146:**471-484.

103. Rhee SS, Marsh JW: **Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4.** *J Virol* 1994, **68:**5156-5163.

104. Luo T, Anderson SJ, Garcia JV: **Inhibition of Nef- and phorbol ester-induced CD4 degradation by macrolide antibiotics.** *J Virol* 1996, **70:**1527-1534.

105. Fredericksen BL, Wei BL, Yao J, Luo T, Garcia JV: **Inhibition of endosomal/lysosomal degradation increases the infectivity of human immunodeficiency virus.** *J Virol* 2002, **76:**11440-11446.

106. Este JA, Cihlar T: **Current status and challenges of antiretroviral research and therapy.** *Antiviral Res* 2010, **85:**25-33.

107. Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, Pomerantz RJ: **The challenge of finding a cure for HIV infection.** *Science* 2009, **323:**1304-1307.

108. Dahl V, Josefsson L, Palmer S: **HIV reservoirs, latency, and reactivation: prospects for eradication.** *Antiviral Res* 2010, **85:**286-294.

109. Deeks SG: **HIV: How to escape treatment.** *Nature* 2011, **477:**36-37.

110. Mercer J, Schelhaas M, Helenius A: **Virus entry by endocytosis.** *Annu Rev Biochem* 2010, **79:**803-833.

111. Moncunill G, Armand-Ugon M, Clotet-Codina I, Pauls E, Ballana E, Llano A, Romagnoli B, Vrijbloed JW, Gombert FO, Clotet B, et al: **Anti-HIV activity and resistance profile of the CXC chemokine receptor 4 antagonist POL3026.** *Mol Pharmacol* 2008, **73:**1264-1273.

112. Blanco J, Barretina J, Clotet B, Este JA: **R5 HIV gp120-mediated cellular contacts induce the death of single CCR5-expressing CD4 T cells by a gp41-dependent mechanism.** *J Leukoc Biol* 2004, **76:**804-811.

113. Clavel F, Charneau P: **Fusion from without directed by human immunodeficiency virus particles.** *J Virol* 1994, **68:**1179-1185.

114. Este JA, Witvrouw M, Tu J, Desmyter J, De Clercq E, Vandamme AM: **Inhibition of HIV type 1 Tat-mediated trans-activation by oncostatin M in HLtat cells.** *AIDS Res Hum Retroviruses* 1995, **11:**1355-1358.

115. Gonzalez-Ortega E, Mena MP, Permanyer M, Ballana E, Clotet B, Este JA: **ADS-J1 inhibits HIV-1 entry by interacting with gp120 and does not block fusion-active gp41 core formation.** *Antimicrob Agents Chemother* 2010, **54:**4487-4492.

116. Gonzalez E, Ballana E, Clotet B, Este JA: **Development of resistance to VIR-353 with cross-resistance to the natural HIV-1 entry virus inhibitory peptide (VIRIP).** *AIDS* 2011, **25:**1557-1583.

117. Witvrouw M, Schmit JC, Van Remoortel B, Daelemans D, Este JA, Vandamme AM, Desmyter J, De Clercq E: **Cell type-dependent effect of sodium valproate on human immunodeficiency virus type 1 replication in vitro.** *AIDS Res Hum Retroviruses* 1997, **13:**187-192.

118. Phillips DM: **The role of cell-to-cell transmission in HIV infection.** *AIDS* 1994, **8:**719-731.

119. Sato H, Orenstein J, Dimitrov D, Martin M: **Cell-to-cell spread of HIV-1 occurs within minutes and may not involve the participation of virus particles.** *Virology* 1992, **186:**712-724.

120. Ahn KS, Ou W, Silver J: **Inhibition of certain strains of HIV-1 by cell surface polyanions in the form of cholesterol-labeled oligonucleotides.** *Virology* 2004, **330:**50-61.

121. Konopka K, Stamatatos L, Larsen CE, Davis BR, Duzgunes N: **Enhancement of human immunodeficiency virus type 1 infection by cationic liposomes: the role of CD4, serum and liposome-cell interactions.** *J Gen Virol* 1991, **72 (Pt 11):**2685-2696.

122. Mellors JW, Rinaldo CR, Jr., Gupta P, White RM, Todd JA, Kingsley LA: **Prognosis in HIV-1 infection predicted by the quantity of virus in plasma.** *Science* 1996, **272:**1167-1170.

123. Ugolini S, Moulard M, Mondor I, Barois N, Demandolx D, Hoxie J, Brelot A, Alizon M, Davoust J, Sattentau QJ: **HIV-1 gp120 induces an association between CD4 and the chemokine receptor CXCR4.** *J Immunol* 1997, **159:**3000-3008.

124. Iyengar S, Hildreth JE, Schwartz DH: **Actin-dependent receptor colocalization required for human immunodeficiency virus entry into host cells.** *J Virol* 1998, **72:**5251-5255.

125. Jimenez-Baranda S, Gomez-Mouton C, Rojas A, Martinez-Prats L, Mira E, Ana Lacalle R, Valencia A, Dimitrov DS, Viola A, Delgado R, et al: **Filamin-A regulates actin-dependent clustering of HIV receptors.** *Nat Cell Biol* 2007, **9:**838-846.

126. Barrero-Villar M, Cabrero JR, Gordon-Alonso M, Barroso-Gonzalez J, Alvarez-Losada S, Munoz-Fernandez MA, Sanchez-Madrid F, Valenzuela-Fernandez A: **Moesin is required for HIV-1-induced CD4-CXCR4 interaction, F-actin redistribution, membrane fusion and viral infection in lymphocytes.** *J Cell Sci* 2009, **122:**103-113.

127. Xu X, Guo J, Vorster P, Wu Y: **Involvement of LIM kinase 1 in actin polarization in human CD4 T cells.** *Commun Integr Biol* 2012, **5:**381-383.

128. Gordon-Alonso M, Rocha-Perugini V, Alvarez S, Ursa A, Izquierdo-Useros N, Martinez-Picado J, Munoz-Fernandez MA, Sanchez-Madrid F: **Actin-binding protein drebrin regulates HIV-1-triggered actin polymerization and viral infection.** *J Biol Chem* 2013.

129. Gordon-Alonso M, Sala-Valdes M, Rocha-Perugini V, Perez-Hernandez D, Lopez-Martin S, Ursa A, Alvarez S, Kolesnikova TV, Vazquez J, Sanchez-Madrid F, Yanez-Mo M: **EWI-2 association with alpha-actinin regulates T cell immune synapses and HIV viral infection.** *J Immunol* 2012, **189:**689-700.

130. Gordon-Alonso M, Rocha-Perugini V, Alvarez S, Moreno-Gonzalo O, Ursa A, Lopez-Martin S, Izquierdo-Useros N, Martinez-Picado J, Munoz-Fernandez MA, Yanez-Mo

M, Sanchez-Madrid F: **The PDZ-adaptor protein syntenin-1 regulates HIV-1 entry.** *Mol Biol Cell* 2012, **23:**2253-2263.

131. Garcia-Exposito L, Ziglio S, Barroso-Gonzalez J, de Armas-Rillo L, Valera MS, Zipeto D, Machado JD, Valenzuela-Fernandez A: **Gelsolin activity controls efficient early HIV-1 infection.** *Retrovirology* 2013, **10:**39.

132. Permanyer M, Ballana E, Badia R, Pauls E, Clotet B, Este JA: **Trans-infection but not infection from within endosomal compartments after cell-to-cell HIV-1 transfer to CD4+ T cells.** *J Biol Chem* 2012, **287:**32017-32026.

133. Blanco J, Barretina J, Cabrera C, Gutierrez A, Clotet B, Este JA: **CD4(+) and CD8(+) T cell death during human immunodeficiency virus infection in vitro.** *Virology* 2001, **285:**356-365.

134. Moncunill G, Armand-Ugon M, Pauls E, Clotet B, Este JA: **HIV-1 escape to CCR5 coreceptor antagonism through selection of CXCR4-using variants in vitro.** *AIDS* 2008, **22:**23-31.

135. Downey GP, Chan CK, Lea P, Takai A, Grinstein S: **Phorbol ester-induced actin assembly in neutrophils: role of protein kinase C.** *J Cell Biol* 1992, **116:**695-706.

136. Vorster PJ, Guo J, Yoder A, Wang W, Zheng Y, Xu X, Yu D, Spear M, Wu Y: **LIM kinase 1 modulates cortical actin and CXCR4 cycling and is activated by HIV-1 to initiate viral infection.** *J Biol Chem* 2011, **286:**12554-12564.

137. Cameron PU, Saleh S, Sallmann G, Solomon A, Wightman F, Evans VA, Boucher G, Haddad EK, Sekaly RP, Harman AN, et al: **Establishment of HIV-1 latency in resting CD4+ T cells depends on chemokine-induced changes in the actin cytoskeleton.** *Proc Natl Acad Sci U S A* 2010, **107:**16934-16939.

138. Mondor I, Ugolini S, Sattentau QJ: **Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans.** *J Virol* 1998, **72:**3623-3634.

139. Saphire AC, Bobardt MD, Zhang Z, David G, Gallay PA: **Syndecans serve as attachment receptors for human immunodeficiency virus type 1 on macrophages.** *J Virol* 2001, **75:**9187-9200.

140. de Witte L, Bobardt M, Chatterji U, Degeest G, David G, Geijtenbeek TB, Gallay P: **Syndecan-3 is a dendritic cell-specific attachment receptor for HIV-1.** *Proc Natl Acad Sci U S A* 2007, **104:**19464-19469.

141. Zaitseva M, Romantseva T, Manischewitz J, Wang J, Goucher D, Golding H: **Increased CXCR4-dependent HIV-1 fusion in activated T cells: role of CD4/CXCR4 association.** *J Leukoc Biol* 2005, **78:**1306-1317.

142. Sloan RD, Kuhl BD, Mesplede T, Munch J, Donahue DA, Wainberg MA: **Productive Entry of HIV-1 during Cell-to-Cell Transmission via Dynamin-Dependent Endocytosis.** *J Virol* 2013, **87:**8110-8123.

143. Kramer VG, Schader SM, Oliveira M, Colby-Germinario SP, Donahue DA, Singhroy DN, Tressler R, Sloan RD, Wainberg MA: **Maraviroc and other HIV-1 entry inhibitors exhibit a class-specific redistribution effect that results in increased extracellular viral load.** *Antimicrob Agents Chemother* 2012, **56:**4154-4160.

144. Zhong P, Agosto LM, Ilinskaya A, Dorjbal B, Truong R, Derse D, Uchil PD, Heidecker G, Mothes W: **Cell-to-cell transmission can overcome multiple donor and target cell barriers imposed on cell-free HIV.** *PLoS One* 2013, **8:**e53138.

145. Duncan CJ, Russell RA, Sattentau QJ: **High multiplicity HIV-1 cell-to-cell transmission from macrophages to CD4+ T cells limits antiretroviral efficacy.** *AIDS* 2013, **27:**2201-2206.

146. Davis HE, Morgan JR, Yarmush ML: **Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes.** *Biophys Chem* 2002, **97:**159-172.

147. Gervaix A, West D, Leoni LM, Richman DD, Wong-Staal F, Corbeil J: **A new reporter cell line to monitor HIV infection and drug susceptibility in vitro.** *Proc Natl Acad Sci U S A* 1997, **94:**4653-4658.

148. Komarova NL, Levy DN, Wodarz D: **Synaptic transmission and the susceptibility of HIV infection to anti-viral drugs.** *Sci Rep* 2013, **3:**2103.

149. Mazurov D, Ilinskaya A, Heidecker G, Lloyd P, Derse D: **Quantitative comparison of HTLV-1 and HIV-1 cell-to-cell infection with new replication dependent vectors.** *PLoS Pathog* 2010, **6:**e1000788.

150. Groot F, van Capel TM, Schuitemaker J, Berkhout B, de Jong EC: **Differential susceptibility of naive, central memory and effector memory T cells to dendritic cell-mediated HIV-1 transmission.** *Retrovirology* 2006, **3:**52.

ACKNOWLEGMENTS

Finalment només em queda donar les gràcies a tothom que ha fet possible que avui pugui estar escrivint aquesta pàgina.

En primer lloc agrair al José Esté per haver-me donat la oportunitat de realitzar aquesta tesi, per haver confiat en mi, per haver-me iniciat i instruït en el món de la ciència.

En segon lloc agrair a la gent del meu grup, a l'Alba, al Roger i a l'Eva amb els quals em sento part d'una família. En especial agrair a l'Ester i a l'Edu, per la paciència, el recolzament, el coneixement i l'autoestima que heu sabut transmetre. També al Gerard perquè ha sigut curt però intens. Tampoc m'oblidaré del "turisteo" per Bulgaria amb l'Ester i l'Alba (entre xerrades del congrés clar...), ni dels dinars, ni dels cafés post-reunió, ni dels "entrenus" amb l'Edu i el Roger, ni de les botifarres a Olesa en família, ni d'altres petits però tant necessaris bons moments que hem compartit durant tot aquest temps. Sou tots molt grans. Igualment agrair també a tota la gent que ja no hi són però que han format part de JAE en algun moment d'aquest 4 anys; Mercedes, Emmanuel, Cristina, Mari Pau...

També vull agrair a tota la gent que forma o ha format part d'Irsi durant aquest 4 anys. Sou tots una gran família, em sento molt afortunat d'haver pogut compartir aquest temps i dubto que em torni a trobar en algun altre lloc amb tanta gent, tant maca i tant ben avinguda. Especialment també a en Bonaventura Clotet per liderar aquest gran projecte. Gràcies a tots.

No vull deixar-me a la gent externa a Irsi però que han contribuït durant algun moment de la tesi; al servei de microscòpia de la UAB, i a la gent del IGTP com la Pilar i en especial al Marco i el Gerard del servei de citometria que crec que han fet possible part important d'aquest treball.

També vull agrair a la família, als meus pares per la paciència, a la Marina per la comprensió, a l'Oriol per treure'm a "passejar" durant les tardes interminables d'agost de tesi, i en general a tots aquells amics i companys que d'alguna manera heu fet oblidar-me momentàniament de tanta ciència.

Durant aquest 4 anys he tingut la oportunitat de conèixer gent fabulosa, amb ganes de treballar, compartir, aprendre, i créixer. Em sento que m'he enriquit personalment i professionalment. Ara és moment de passar pàgina, de continuar endavant en aquest camí tant difícil com enriquidor que és la ciència.

Gràcies a tots, sense vosaltres no hauria estat el mateix.