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On the Steps of Cell-to-Cell HIV Transmission

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SUMMARY

Unlike virus-to-cell contacts, cell-to-cell HIV transmission (from infected cells to CD4 T cells) is a highly efficient and cytopathic mechanism. Such a high efficiency relies in the formation of "virological synapses" induced by the HIV envelope glycoproteins. The particularity of this synapse as compared to the classical ones would be that information is transferred as virus particles (accompanied by other components of the cell membrane) from effector to target cells. First, the aim was to characterize T cell-T cell contacts mediated by the HIV Envelope in mixed cultures of infected and target primary cells expressing or not the appropriate coreceptor. Then we have evaluated HIV transfer, which is an early event occurring immediately after the VS formation that precedes but does not necessarily lead to transmission, a later event resulting in infection. The presence of adhesion molecules at the synaptic junction showed a secondary role in cell-to-cell HIV transfer. Despite memory CD4 T cells expressed higher levels of adhesion molecules that naïve cells, it did not explain the selectivity of HIV transfer observed towards the memory CD4 T cell subset. Trogocytosis (i.e. intercellular exchange of membrane components) operates from the uninfected towards the infected cell direction but may modulate the extent and durability of HIV-mediated T cell contacts. Finally, cell-to-cell HIV transmission triggers the infection of quiescent CD4 T cells, which showed low levels of Envelope-CD4 mediated p56Lck and ZAP-70 phosphorylation in the absence of proliferation and productive infection of target cells.

RESUM

A diferència de la infecció per virus lliure, la transmissió del VIH de cèl·lula a cèl·lula és un mecanisme altament eficient i citopàtic degut a l'establiment de les sinapsis virològiques induïdes per l'embolcall del virus on la informació es transmet en forma de partícules virals (acompanyades de traços de membrana) des de la cèl·lula efectora a la cèl·lula diana. L'objectiu inicial va ser la caracterització dels contactes cel·lulars mediats pel VIH en cultius mixtes de cèl·lules infectades i cèl·lules no infectades en absència o presència del coreceptor apropiat. Posteriorment es va evaluar la transferència viral de cèl·lula T a cèl·lula T, un fenòmen que pot o no portar a la infecció de la cèl·lula diana. Les molècules d'adhesió tenen un paper secundari en la transmissió viral a través de la sinapsis virològica. Tot i que les cèl·lules T de memòria expressen més quantitats d'aquestes molècules que les cèl·lules T naïve, la major transferència viral observada cap a aquestes no es pot explicar per aquest fet. La trogocitosis o intercanvi de components de membrana, no permetla transferència de virus ja que només opera en el sentit invers a la transmissió tot i que podria estar jugant un paper modulador en la formació i durada dels contactes. Finalment, la transmissió viral de cèl·lula a cèl·lula desencadena la infecció de cèl·lules T CD4 quiescents, les quals presenten nivells baixos de fosforilació de les proteïnes senyalitzadores p56Lck i ZAP70 un cop han contactat amb les cèl·lules infectades, en absència de proliferació cel·lular i infecció productiva d'aquestes cèl·lules en estat en repòs, permanent així, en un estat latentment infectades.

RESUMEN

A diferencia de la infección por virus libre, la transmisión de VIH de célula a célula es un mecanismo altamente eficiente y citopático debido al establecimiento de las sinapsis virológicas inducidas por la envuelta del virus donde la información se transmite en forma de partículas virales (acompañadas de trazos de membrana) desde la célula efectora a la célula diana. Inicialmente se caracterizó los contactos celulares mediados por el VIH en cultivos mixtos de células infectadas y células no infectadas en ausencia o presencia del coreceptor apropiado y posteriormente se evaluó la transferéncia viral. Las moleculas de adhesion juegan un papel secundario en la transmisión viral a través de la sinapsis virológica. Las células T de memoria expresan más cantidades de estas moléculas que las células T naive, aún así, la mayor transferencia viral observada hacia as células de memoria no se explica por este hecho. La trogocitosis o intercambio de componentes de membrana, no permite la transferéncia de virus ya que sólo opera en el sentido inverso a la transmisión aunque puede tener un papel modulador en la formación y durada de los contactos. Finalmente, la transmisión viral de célula a célula desencadena la infección de células T CD4 quiescentes, las cuales presentan niveles bajos de fosforilación de las proteínas p56Lck y ZAP70 un vez han contactado con las células infectadas, en ausencia de proliferación celular y infección productiva de estas células en estado de reposo, permaneciendo así, en un estado latentemente infectadas.

ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen presenting cell
APOBEC3G	Apoliprotein B mRNA editing enzme catalytic polypeptide-like 3G
CAp24	HIV antigen capsid p24
CMFDA	CellTracker™ Green, 5-chloromethyl fluorescein diacetate
CMRA	CellTracker™ Orange
CCR5	Chemokine receptor 5, also known as CD195
cSMAC	Central supramolecular activation cluster
CTL	Cytotoxic T lymphocyte
CXCR4	CXC Chemockine receptor 4, lso known as CD184
DC	Dendritic Cell
DDAO	CellTrace™ Far Red
DiO	3,3'-dioctadecyloxacarbocyanine perchlorate, Molecular Probes
DiI	1,1' - Dioctadecyl - 3,3,3',3' tetramethyl-indocarbocyanine iodide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide
ELISA	Enzyme -Linked ImmunoSorbent Assay
Env	HIV envelope glycoprotein
ERM	Ezrin-radixin-moesin
Fab	The fragment antigen-binding
FACS	Flow cytometry, acronym from Becton Dickinson
FBS	Foetal bovine serum
FcR	Receptor of the fragment crystallizable of an antibody
FITC	Fluorescein isothiocyanate
FISH	Fluorescence in situ hybridization
FSC	Forward Scatter
GALT	Gut Associated Lymphoid Tissue
GFP	Green Fluorescence Protein

HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HLA-DR	Human Leukocyte Antigen- DR
HTLV-1	Human T lymphotropic Virus type-1
ICAM-1,-2,-3	Intracellular Adhesion Molecule-1,-2,-3
IgG	Immunoglobulin type G
IL-2	Interleukine-2
IN	HIV integrase enzyme
IP	Propidium iodide
IS	Immunological synapse
ITAMs	Immunoreceptor tyrosine-based activation motif
LAD-1	Leukocyte adhesion deficiency type-1
LFA-1	Lymphocyte Function Associated Antigen-1
LTR	Long Terminal Repeat
MA	HIC matrix protein
mAbs	Monoclonal antibodies
MHC	Major Histocompatibility Complex
MFI	Mean Fluorescence Intensity
MOI	Multiplicity of infection
mRNA	Messenger RNA
MTOC	Microtubule Organizing Centre
nAbs	Neutralizing antibodies
NC	HIV nucleocapsid
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institute of health
PBMCs	Peripheral Blood Mononuclear cells
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PIC	Preintegration complex
Pol	HIV polymerase enzyme
PR	HIV protease enzyme
pSMAC	Peripheral supramolecular activation cluster

qCD4	Quiescent CD4 T cells
qPCR	Quantitative real-time PCR
Rev	Regulator of virion protein expression
RFI	Realtive Fluorescence Intesity
RNA	Ribonucleic acid
RRE	Rev response element
RT	HIV reverse transcriptase enzyme
SSC	Side Scatter
TAR	Tat activation region
Tat	Trans-activator of transcription
TCID50	Median tissue culture infective dose
TCR	T Cell Receptor
VS	Virological synapse
VSV	Vesicular Stomatitis Virus
Wt	Wilde-type

CONTENTS

INTRODUCTION

1. The human immunodeficiency virus type 1 (HIV) infection

The human immunodeficiency virus (HIV) belongs to the family of animal retroviruses characterized for their enveloped structure surrounding a viral core of single-stranded RNA. HIV is the agent causing the acquired immunodeficiency syndrome (AIDS), characterized by a loss of the immune function, which in turn correlates with the appearance of opportunistic infections due to the lack of immune protection. HIV is transmitted between human's body fluids where HIV is present as both free virus particles or associated with immune cells.

1.1 HIV and the Immune system

At the beginning of the infection, HIV cross the mucosal barriers; the first host defense, by mechanisms that may or may not involve infection of the cells present in the mucosal tissues such as dendritic cells (DC) and macrophages. DCs that have captured the virus migrate to the nearest lymph nodes and present antigens and infectious viruses to specific CD4 T cells. Within days after the first exposure, viral replication can be detected in the lymph nodes and this is translated into an increase in the viral burden observed in the plasma of the infected individuals (i.e. viremia) (Figure 1). Then, both humoral and cell-mediated immune responses are mounted, a feature that accounts for a decrease in viral load to still detectable levels (Figure 1). During the early stages of the HIV infection,

memory CD4 T cells in mucosal lymphoid tissues (i.e. gut associated lymphoid tissue, GALT) are the principal target of HIV infection and subsequent cell death. In the next, chronic phase of the disease, lymph nodes and the spleen are sites of continuous HIV replication and cell destruction. It has been suggested that more than 90% of the body's infected CD4 T cells are normally found in peripheral and mucosal lymphoid tissues. Early in the course of the disease, the body may continue to make new CD4 T cells, which can be replaced almost as quickly as they are destroyed until the regenerative capacity of the thymus is slowly destroyed and the minimal number of CD4 T cells necessary to maintain an immune response is lost, leading to the acquired immune deficiency syndrome or AIDS.

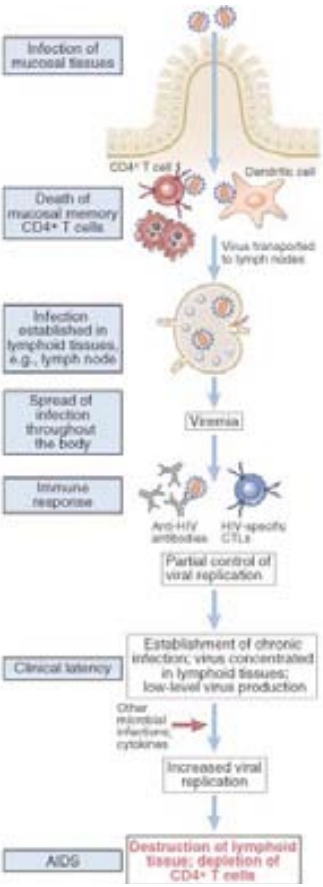


Figure 1. Progression of HIV infection. The progression of HIV infection correlates with spread of the virus from the initial site of infection to lymphoid tissues throughout the body. The immune response of the host temporarily controls acute infection but does not prevent the establishment of chronic infection of cells in lymphoid tissues. Cytokine stimuli induced by other microbes serve to enhance HIV production and progression to AIDS. Illustration from Elservier.Abbas et al: Cellular and molecular immunology 6th.

1.2The HIV life cycle

The HIV genome encodes nine open reading frames; three of these encode the Gag, Pol (polymerase), and Env (envelope) polyproteins, which are subsequently proteolyzed into individual proteins common to all retroviruses; the four Gag proteins: MA (matrix), CA (capsid), NC (nucleocapsid), and p6, and the two Env proteins: gp120 and gp41 are structural components, the three Pol proteins; PR (protease), RT (reverse transcriptase), and IN (integrase), provide essential enzymatic functions and are also encapsulated within the particle. HIV encodes six additional proteins, often called accessory proteins, three of which (Vif, Vpr, and Nef) are found in the viral particle. Two other accessory proteins, trans-activator of transcription (Tat) and regulator of virion protein expression (Rev), provide essential gene regulatory functions, and the last protein, Vpu, indirectly assists in assembly of the virion. The retroviral genome is encoded by 9-kb RNA organized in two identical RNA molecules which are also packaged in the particle. The sequences of events that take place each time a new target cell gets infected are illustrated in Figure 2:

1. *Viral attachment to cell surface receptors;* Long ago it was identified that HIV enters its target cell through the interaction of the Env glycoprotein with the receptor CD4 expressed on the surface of T lymphocytes and to a lesser extent in macrophages and dendritic cells [1, 2].

2. *Fusion of viral and cellular membranes, entry of viral core into cell cytoplasm;* CD4 alone is not sufficient to permit entry of the viral genome inside the target cell, and a second interaction with the appropriate chemokine receptor (CXCR4, CCR5) is further required.

3. *Uncoating of the viral core;* This process involves the release of the genetic HIV material and other viral proteins into the cytoplasm of the target cell.

4. *Reverse transcription of viral genome*; the two single-stranded RNAs that virions carry are reverse transcribed into mainly a double-stranded cDNA (dscDNA) by the HIV RT. Then the dscDNA forms a complex with various viral and host proteins that has been termed pre-integration complex (PIC).

5. *Microtubule-mediated movement of PIC towards the nucleus followed by nuclear import across the nuclear pore*

6. *Integration of proviral genomic DNA into the host chromatin;* Inside the nucleus, the viral integrase enzyme catalyzes the integration of the viral DNA into the host cell genome by binding to the long terminal repeat (LTR) regions located at both ends of the HIV DNA strand. When HIV is integrated, it is referred as the "provirus" although it can also remain as unintegrated DNA, a form that may represent almost 99% of the total DNA that has entered the nucleus [3].

7. *Transcription of provirus in activated cells and export of spliced and unspliced mRNA;* Transcription of the integrated provirus is dependent on the host cell RNA polymerase II transcription machinery, but is regulated in critical ways by the Tat and Rev proteins with the help of numerous cellular transcription factors that are present in activated T cells. In the early phase of viral transcription, a multiply-spliced set of mRNAs is generated, producing the transcripts of the regulatory proteins, Tat, Rev, and Nef. The Tat protein is an RNA specific trans-activator of LTR-mediated transcription. Transcription of the provirus starts within the HIV LTR found in the 5′ end of the DNA.

8. *Assembly of viral proteins and genome;* When transcription is completed, a "full-length" viral RNA is generated as a non-spliced transcript that will serve as a messenger for the newly synthesized structural HIV proteins (Gag and Pol) as well as the new genomic RNA. Later a set of spliced
and genomic-length RNAs are exported from the nucleus to the cytoplasm in a step regulated by Rev. The viral mRNAs are translated into viral proteins in the cytoplasm, where the Gag and Gag-Pol polyproteins travel to the cell membrane across the cellular microtubule network. The Env mRNA is translated at the endoplasmic reticulum and the protein is then expressed on the surface of infected cells.

9. *Viral budding, release and maturation;* the core particle is assembled and an immature virion begins to bud from the cell surface dragging the Env glycoprotein as well as other cell-surface molecules. As the particle buds and is released from the cell surface, the virion undergoes a morphologic change known as maturation with the help of the HIV PR enzyme that is responsible for cleaving the HIV polyprotein.

Aside from the common HIV life cycle previously exposed, other routes of HIV infection need to be considered. Since HIV viral particles *in vivo* present a short lifespan, HIV, like other retroviruses, has overcome this limitation by spreading between cells. Early studies highlighted the increased efficiency of HIV spread from cell-to-cell as compared with fluid-phase diffusion [1-4]. Replication of HIV in primary human CD4 T lymphocytes is strictly coupled to the activation state of these target cells (reviewed in [5]). However, *in vivo*, CD4 T cells, which are normally found in a non-dividing or resting state, have been found latently infected. In this state, the virus is unaffected by immune responses as it prevails as a latent reservoir inside quiescent CD4 T cells (qCD4) thus becoming a major barrier for the final eradication of the infection.

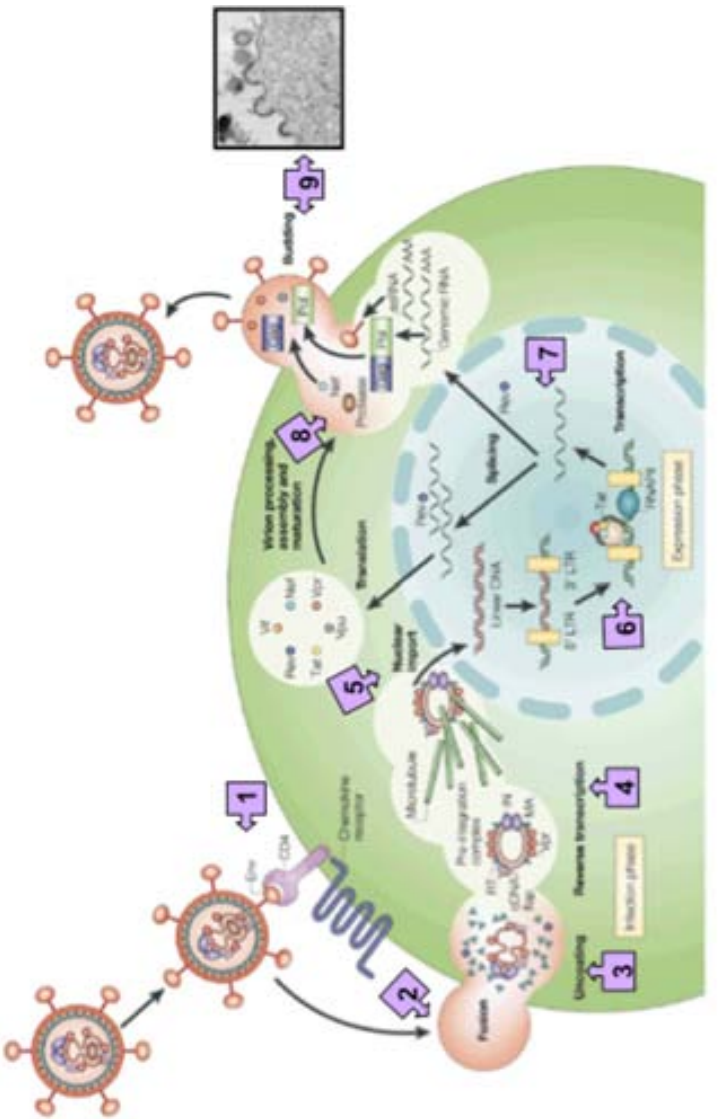


Figure 2. The replicative cycle of HIV. Illustration adapted from [6]

1.3 The role of the HIV Env and its receptors

Entry of HIV into target cells is mediated by the interaction of the cell surface CD4 receptor and the appropriate coreceptor with the Env glycoprotein gp120. HIV infection commonly leads to the disappearance of CD4 from the plasma membrane, a phenomenon known as down-modulation/down-regulation, which renders cells refractory to subsequent infections with the same virus. This phenomenon is essential for the survival and replication of the virus [7]. Down-modulation also occurs during antigen presentation where the TCR-peptide loaded MHC complex are internalized inside the T cell in order to stop the antigen presentation and subsequent stimulation process.

The binding of HIV Env to either the CCR5 or CXCR4 coreceptors determine the tropism of the virus (R5 or X4, respectively), by means of the target cell type they are capable of infecting [8, 9]. Dual tropic R5X4 isolates are capable of fusing either CCR5 or CXCR4 expressing cells. In most cell-culture models for HIV infection, X4 isolates appear to be more pathogenic than R5 isolates [10], and *in vivo*, X4 viruses have been associated with a faster disease progression and poor prognosis [11]. This fact could be explained by the higher expression of CXCR4 in the surface of T lymphocytes as compared to CCR5, thus X4 viruses present a wider spectrum of infection than the R5 viruses *in vivo*. Nonetheless, R5 isolates are also

able to deplete CD4 T cells and cause AIDS in HIV-infected patients and CCR5 has been identified as the major entry cofactor for most

primary or clinical isolates of HIV [12]. In addition, other chemokine receptors can function as coreceptors of HIV entry *in vitro* but their role *in vivo* is questionable [13-16].

During the process of viral entry, the Env gp120 subunit expressed either on the surface of infected cells or on the surface of viral particles, interacts with its receptor CD4 expressed on the surface of target cells. This first interaction triggers a structural change in the Env conformation resulting in the exposure of the coreceptor-binding site of the gp120 subunit. Afterwards, coreceptor-mediated interaction may trigger a complex sequence of events explained as follows:

Cell fusion/hemifusion: Infected cells or cell-free viruses fuse with uninfected CD4 T cells expressing the appropriate coreceptor [17, 18]. Fusion of the viral and the cellular membranes mediates the formation of a large *fusion pore* that enables the release of the HIV nucleocapsid into the cytoplasm of the target cell [16] to undergo the infectious process[14] (Figure 3A). However, in the context of infected-uninfected T cell interaction, the outer leaflets of the lipid bilayers that surround the cells in contact may fuse in a process defined as hemifusion. This fusion event, prior to the formation of the fusion pore, is characterized by the mixing of membrane lipids without intracellular content mixing [19] (Figure 3A). Depending on

the *in vitro* or *in vivo* conditions (i.e. levels of fusion protein or coreceptor expression), the process may either stop at a hemifusion-

like event or proceed to complete fusion. *In vitro*, when the process involves the fusion of Env-expressing cells with target CD4 T cells, the effect may result in the formation of a giant multinucleated cell acknowledge as *syncitium* which finally lead to multiple cell death (Figure 3B).

Cell death: In vivo, T cell depletion far exceeds the number of infected T cells suggesting an indirect mechanism of HIV pathogenesis into bystander cells [16]. Besides, in lymph node sections of HIV-infected patients it appears that the majority of apoptotic cells are uninfected cells found in close proximity to infected cells [20]. Depending on Env presentation and on the complexity of target cell contact, the mechanisms leading to cell death may also be different. Indeed, soluble Env, secreted from infected cells, Env expressed on virions or at the cell surface of infected cells, are able to induce apoptosis of uninfected bystander T cells [21] (Figure 3B). Numerous *in vitro* studies demonstrated the apoptotic role of Env-coreceptor-mediated interactions [21-24] and its inhibition using antagonists such as AMD3100 (anti-CXCR4) or the fusion inhibitor peptide C34 [25, 26]. The formation of syncitia is rarely observed *in vivo* except in brain and tonsils [21], thus, the fusion process may be interrupted at an hemifusion level but still death of the bystander cells is observed [25] (Figure 3B). To corroborate this hypothesis, apoptosis was

tested in bystander T cells from *in vitro* experiments with the Env mutant D589L that is restricted at the hemifusion step, and observed a new role of Env gp41 subunit in fusion-independent pathogenic events [16]. This mechanism of bystander cell death mediated via Env-coreceptor interaction shows classical signs of apoptosis which have been associated to caspase-3 activation, mitochondrial depolarization as well as reactive oxygen species production [27, 28] although more recently, a link with autophagy has been demonstrated [29, 30] (reviewed in [31]). Autophagy is a catabolic process involving the degradation of a cell's own components through the lysosomal machinery. HIV-infected cells can induce autophagy into bystander CD4 T lymphocytes through contact of Env to coreceptor but not the CD4 receptor, leading to apoptotic cell death [29].

Infection: Upon coreceptor binding, the fusion of the viral and cell membranes allows for the release of the HIV genetic material into the target cell, (i.e.uncoating) whereby the infectious process starts [13-15].

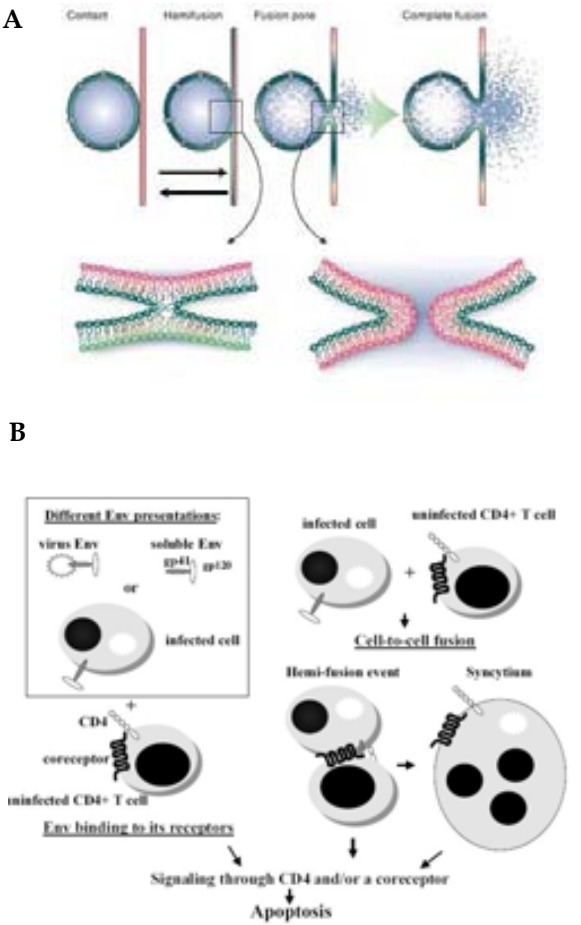


Figure 3. T**he sequence of events mediated by Env-CD4/coreceptor interaction A.** HIV cell-to-cell membrane fusion is likely to proceed through hemifusion. The latter process can revert back to the single cell stage and lead to Env-mediated cell death. Illustration adapted from [32]. **B.** Schematic diagram of Env-induced CD4 T cell apoptosis. Either cell-free HIV particles or cell-mediated HIV interaction with target cells may lead to final apoptosis through Env-CD4/coreceptor mediated signaling [21].

1.4 Selectivity of HIV infection towards different CD4 T cell subsets

There is an extensive diversity displayed by CD4 T cells in terms of phenotype, function, and anatomical distribution. The cellular subtypes are heterogeneous and can be subdivided into naïve and memory subsets according to the differential expression of the CD45RA or CD45RO antigens, respectively (reviewed in [33]). Naïve T cells exit the thymus and enter into the bloodstream to reach lymphoid tissues where antigen presentation takes place. Naïve T cells remain in a G0 state and so require a stimulation of DCs exposing the related antigen to be able to proliferate [34]. Cells receiving a weak stimulation die by apoptosis, whereas those receiving a strong stimulation become effector cells or enter the memory pool. The memory subset remains in a non-dividing (quiescent) state and has a higher sensitivity to antigenic stimulation compared to naïve cells [34]. It is known that R5 viruses are primarily transmitted between individuals and that X4 viruses emerge only later in infection [35, 36] (Figure 4). An increasing number of studies on HIV demonstrate that the initial burst of viral replication takes place in CD4/CCR5 positive memory T cells and that later in infection, proviral DNA can be isolated from both naïve and memory CD4+ T cells [37, 38]. The CD45RA and CD45RO CD4 T cell subsets showed strong differences in HIV-binding capacity [34, 37], and HIV-mediated cell death [38, 39] despite the fact that the level of CD4 expression (the main receptor for HIV infection) are

similar in both subsets [39]. Our goal was to explore the differences in T cell-T cell HIV transmission to both CD4 T cell subsets.

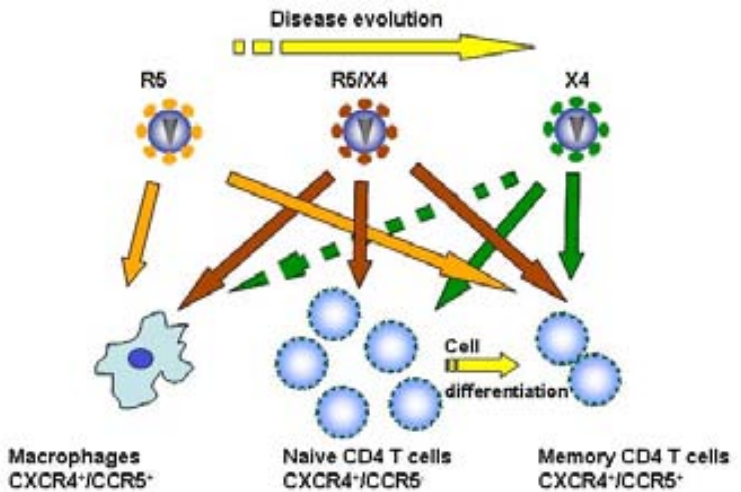


Figure 4. Diagram of HIV disease progression according to coreceptor usage. R5 isolates infect cells of the immune system more accessible at the sites of viral entry (such as macrophages and memory CD4 T cells). X4 isolates normally appear later in the course of an HIV infection and present a wider spectrum of target cells including naïve CD4 T cells mainly found in the lymph nodes.

2 Mechanisms of HIV spread

2.1 Free virus infection vs. cell-to-cell spread

There is growing evidence that a number of different viruses can exploit preexisting mechanisms of physiological communication between cells to facilitate direct cell–cell viral spread [40-42]. Taking advantage of cellular communication has conferred to HIV a clear and substantial benefit over diffusion-limited spread in terms of viral dissemination efficiency [43]. Early studies suggested that cell–cell dissemination might be 100 to 1000 times faster than cell-free HIV spread [1, 4]. This highly efficient mode of HIV spread has been first described between antigen presenting cells (APC), mainly DC, and uninfected CD4 T lymphocytes, while more recently, HIV cell-to-cell spread has been also detected between infected and uninfected CD4 T cells (Figure 5).

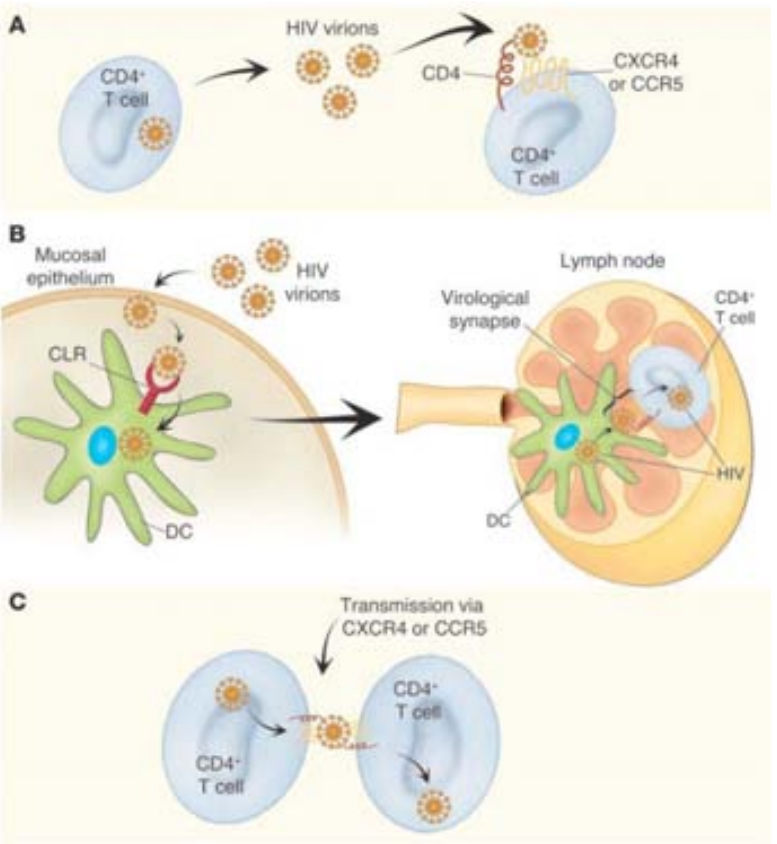


Figure 5. Three mechanisms of HIV propagation. A. Cell-free viral transmission. **B.** DC–T cell viral trans-mission. **C.** T cell–T cell viral transmission. Illustration taken from [44] The Journal of Clinical Investigation.

A more reinforced evidence supporting the cell-to-cell transmission route came from experiments in which the efficacy of HIV spread was dramatically reduced in constantly agitated lymphocyte cultures [45] or by physical separation of effector and target cells in transwell experiments [45, 46]. Additional studies showed confocal images revealing the co-clustering of CD4, CXCR4 and Env at the interface between conjugates of infected and uninfected CD4 T cells and have termed this structure *virological synapse* (VS) [44, 47, 48]. The first detailed description of VS was reported for the human T cell leukemia virus type 1 (HTLV-1). The cell-free form of HTLV-1 is very inefficient at infecting T cells and is spread between, and within, individuals by cell-to-cell mechanisms [49, 50]. The direct passage of virus across the VS is a process that may particularly be important in tissues where cells are much more densely packaged, such as in epithelial or endothelial surfaces, lymphoid organs and neuronal axons [47]. Noteworthy, cell-to-cell viral spread presents numerous advantages for the virus as compared to cell-free virus infections:

- A more rapid replication kinetics is obtained when viruses are transmitted across VS due to the higher concentration of viral particles released at the point of contact between the cell partners thus obviating the rate-limiting step of fluid-phase diffusion of free viral particles [47].

- Infected cells entering a new host could adhere to, and cross by transmigration, a mucosal epithelial barrier that would otherwise be impermeable to cell free virions [42].
- The formation of stable junctions whereby viruses traffic, shields the virus both sterically and kinetically in terms of exposure time [42] although it is still unclear whether cell-cell spread may confer HIV a protection from immune responses and viral entry inhibitors [43].

Despite the benefit of VS, HIV-mediated cellular conjugates induce target cell death. The Env (gp120/gp41) expressed at the surface of infected cells drives cell-to-cell fusion with adjacent uninfected CD4+ T cells, which results in the formation of multinucleated syncytia [21]. Hemifusion events as well as syncytium formation have been shown to trigger cell apoptosis and thus to participate to the global loss of CD4+ T cells during AIDS [21].

2.2 HIV transmission across the VS

2.2.1 Definition, types and functions

The word synapse is derived from Greek, meaning "point of contact" (Oxford English Dictionary) and the term is widely used in the biomedical literature [44]. Synapses always involve a pre and a post-synaptic cell that specifically encounter each other and establish a contact that delivers signals with the attempt to

communicate between them. Four different criteria need to be fulfilled in order to define a functional synapse [51]:

- In a synaptic structure the two synaptic partners should stick together even though remaining as *individual entities* (i.e. membrane surfaces parallel to each other with a fluid-filled space in between) [51].
- The synaptic partners recognize each other and then the pre and post-synaptic surfaces are locked together, a role normally developed by *adhesion molecules* [52].
- The adhesive junction provides *stability* where the cell cytoskeleton plays an important role in the strength and organization of the synapse [51].
- On the pre-synaptic side, a *directed secretion* is assembled whereas on the post-synaptic cell, a clustered receptor surface receives the molecules released and starts the signaling cascade [51].

Many different types of synapses have been described so far; the neural synapse, the immunological synapse (IS), and later the VS, which are discussed in more detail:

A) The *neural synapse* was described 100 years ago and it consists of points of contiguity between neurons across which information (via neurotransmitters) is relayed [44]. Neural synapses provide the means through which the nervous system connects to and controls the other systems of the body. Stability between the interacting neurons is established and maintained by an adhesion molecule scaffold, composed mainly of cadherins [51].

B) A synaptic basis for immune cells' communication was suggested in the early 1980 [53]. The use of the term *immunological synapse* refers to close cell-cell contacts between cells of the immune system. They are based on antigen recognition by the T cell receptor (TCR) and may be formed between cytotoxic CD8 T cells and target cells or between professional APCs (such as DC) and T lymphocytes (either CD4 or CD8 T lymphocytes). DCs reside in the skin and mucosal tissues in an immature state until they encounter pathogen-associated antigens and undergo a series of morphological and functional changes that will finally lead to a mature phenotype of the DC. This maturation is closely linked to the migration of DCs from peripheral tissues to secondary lymphoid organs where they interact with antigen-specific T cells through the establishment of ISs in order to initiate an immune response [44]. Depending on the contact duration, receptor engagement and downstream signaling, a variety of immunological events will take place [54-56]. Long-lasting contacts between APC and T cells may allow for the control of transcriptional activity of genes involved in T cell activation [57].

C) There are three different models in which *virological synapses* have been described depending on the type of effector cells that form the synapse. In the first system, HIV infected cells, when entering the body, will firstly encounter non-immune cells such as those of the mucosal epithelia of the genital or gastrointestinal tracts. These cells are not considered permissive to infection, but may function to transfer virus to underlying lamina propia that is rich in susceptible cells [58] in a process termed *transcytosis* [59]. In the second model, the pre-synaptic cell is a DC or another APCs that captures and internalizes incoming cell-fee virions whereas the postsynaptic cell studied in more detail to date is the CD4 T cell [44]. The term DC encompasses several cellular subpopulations such as Langerhans cells and myeloid DCs, which may play an important role during the early stages of HIV infection, most notably during sexual transmission (reviewed in [44]). Cell-to-cell transmission from DCs to T cells can occur subsequently to productive infection of DCs, or via capture of free-virus that do not productively infect DCs, followed by transmission to adjacent target cells. The latter process is referred as *trans-infection*, which takes places through the establishment of the so called *infectious synapse* [60] and represents the "Trojan horse" model proposed for DCs-mediated infection of target cells [61]. Interestingly, human hepatoma cells are also able to capture HIV and transmit it to target CD4 T cells in *trans* [62]. Finally, the term VS has been given to the structures that arise between productively infected cells such as a T cell [63] or a macrophage [64] and an uninfected target CD4 T cell. As synaptic

partners need to be in close contact, this cell-to-cell spread mechanism would be found especially in lymphoid tissues where CD4 T cells appear in high concentrations.

It is unclear at present how HIV has evolved the cell-to-cell transmission mechanism. It is postulated that either HIV could harness cellular machinery that pre-exists for IS assembly for their own benefit or it modifies other cellular programs in a more sophisticated manner [44]. The VS structure closely resembles the IS formed between cytotoxic T lymphocytes (CTL) and target cells and it mainly differs on the receptor that take part (Figure 6). While IS polarizes the secretion of granules towards the synaptic cleft, retroviruses seem to presumable hijack this general trafficking and secretory mechanisms for their own spread. More details on how T cell-T cell VSs are formed is extended in the following sections and will be further analyzed in this thesis.

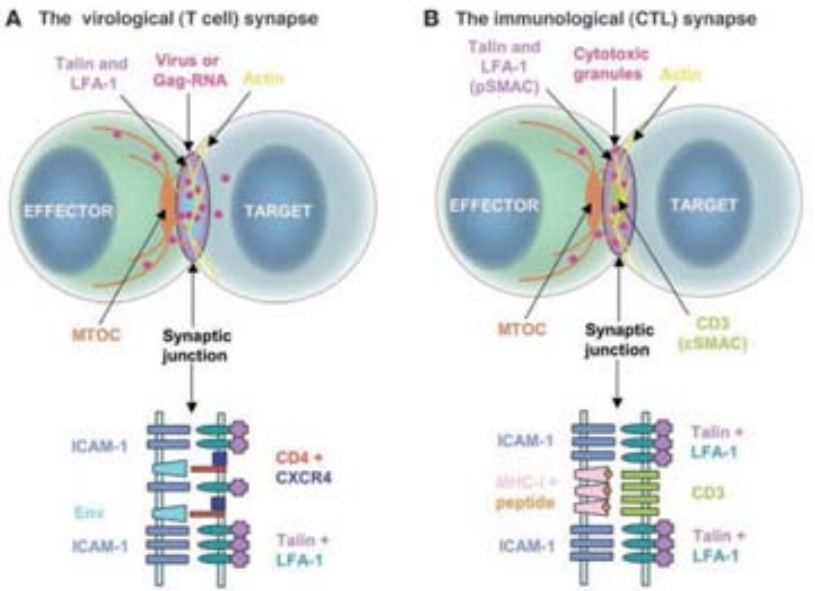


Figure 6. Similarities between the T cell–T cell VS and IS. **A.** In the VS Env ligates CD4 and CXCR4 in the target cell and ICAM-1 engages LFA-1 in the target cell. Gag associated with the viral genome present within the effector cell is recruited along microtubules to the site of cell-cell contact. In the target cell, LFA-1–talin complexes and CD4 and CXCR4 are recruited in an actin-dependent manner to the VS. **B.** In the IS, LFA-1–talin complexes form the pSMAC, whereas the TCR is recruited to the cSMAC, into which cytotoxic granules are secreted in a microtubule-dependent manner. In the target cell, ICAM-1 engages LFA-1 and peptide–MHC-I complexes ligate the TCR of the effector cell. The lower parts of both panels illustrate a simplified schematic of the molecular arrangements present in the VS (A) and IS (B). Illustration from [44] The Journal Clinical of Investigation.

2.2.2 *Synapse structure: cSMAC, MTOC and signaling proteins*

The most studied type of IS is the DC-T cell synapse, which compromises five phases; first cellular scanning, contact acquisition and adhesive arrest, second, early IS-assembly and signaling, third maturation and receptor segregation, fourth, T cell receptor (TCR) - major histocompatibility complex (MHC) internalization and fifth, IS dissolution [56]. As a hallmark of IS formation three basic sets of molecules are found at the IS; surface proteins on the membrane, cytoskeleton and signaling molecules in the cytoplasmatic regions [65].

Surface proteins: The interaction of the TCR on the lymphocytes with the peptide-loaded MHC-class II molecules on the APC form the central ring of the synapse, named central supramolecular activation cluster (cSMAC) that plays a key role in T cell activation [66]. The synapse gains stability through interactions between lymphocyte function-associated adhesion molecule LFA-1 on the T cell to its main ligand intracellular adhesion molecule-1 ICAM-1 on the opposing cell [47], positioned at the peripheral of the IS or pSMAC [67, 68]. LFA-1 is an integrin expressed on the surface of all leukocytes, which mediates cell adhesion, migration and costimulatory signals. The major ligands of LFA-1 are the three intracellular adhesion molecules -1, -2 and -3 (ICAM-1 or CD54, ICAM-2 or CD102 and ICAM-3 or CD50). LFA-1 binds with highest affinity to ICAM-1 followed by ICAM-2 and has the lowest affinity

for ICAM-3. In order to bind to its cognate ligands, LFA-1 needs to unfold and be established in its active conformation. LFA-1 appears to play a key role in the formation of ISs by interacting with its high-affinity ligand ICAM-1. The binding of ICAM-1 to LFA-1 is facilitated by initial low-affinity interactions of LFA-1 with the widely expressed ligand ICAM-3 at the cSMAC [69, 70] that leads to LFA-1 activation and clustering in the periphery of the synaptic structures (pSMAC) stabilizing cellular contacts and providing costimulatory signals [41, 71]. Initial scanning of the APC surface by T cells involves exploratory contacts that are mediated by LFA-1 and their ligands ICAM-1 and ICAM-3, which culminates in specific antigen-loaded MHC class II recognition by the TCR [72, 73]. In this initial scanning LFA-1 is present in the cSMAC linked to the actin cytoskeleton through the protein talin, which would direct the movement of the adhesion molecules out from the cSMAC to the pSMAC. Finally, large and bulk molecules such as CD43 and CD45 are localized in a region distal to the synapse, known as dSMAC [74].

Cytoskeleton molecules: Concomitant to the IS the cytoskeleton-associated proteins actin and tubulin are recruited to this region. Apart from their structural roles, cytoskeleton proteins can also contribute to control the signaling by allowing the different intracellular signaling proteins to position at the site of contact. Likewise, the actin-built cytoskeleton plays an important role in the positioning of the cell surface molecules. In addition, ezrin, radixin

and moesin (ERM) are a family of proteins that connect the actin cytoskeleton to the cytoplasmatic region of several cell-surface proteins mainly located at the pSMAC or dSMAC [73]. It has been observed that in the absence of ERM proteins, the IS was not correctly assembled [75]. During the IS formation, the microtubule-organizing centre (MTOC) and the Golgi apparatus are also oriented to the location underneath the synapse [55, 73].

Signaling molecules: The clustering of signaling molecules at the cytoplasm regions of IS present a role in the regulation of the structure and functions of this region [57]. After the engagement of TCR/CD3-MHC complex, the SRC-family kinase p56Lck is relocalized into lipid rafts and becomes activated [76]. The cytoplasmic domain of CD4 is bound p56Lck, and this complex plays an important role in T-cell activation [77]. Activated p56Lck rapidly engages the SRC-family kinase FYN trough tyrosine phosphorylation [76], and together, p56Lck and FYN phosphorylate consensus sites known as immunoreceptor tyrosine-based activation motifs (ITAMs) that are present in molecules such as ZAP-70 (ζ-chain-associated protein kinase of 70 KDa). This in turn phosphorylates other adaptor molecules and the signaling cascade culminates in the recruitment of actin, actin-anchored receptors and the MTOC to the site of cell-cell contact.

The cross-talk between receptor mediated outside-in signaling, cytoskeleton reorganization and directed transport of cell-surface receptors results in a phenomenon described as “capping” in which cell surface receptors, filamentous actin and lipids congregate towards one end of the cell [74].

2.2.3 *HIV at the T cell-T cell VS*

Although T cell do not usually form homotypic stable interactions, T cell- T cell VSs are formed between productively infected T cells and uninfected CD4 T cells where both X4 [63] and R5 [45, 58, 78] HIV isolates can spread between T cells via this mechanism. T cell-T cell synapses concentrate at the cSMAC the binding of cell surface Env to its receptor CD4 on the uninfected cell [44, 63] (Figure 7). Cellular contacts between infected and uninfected primary CD4 T cells recruits CD4 and coreceptors CXCR4 and CCR5 to the site of cell-cell contact in an actin-dependent manner [63] whereas Env and Gag are recruited to the interface in the effector cell by a microtubule-dependent mechanism [79] finally leading to viral budding towards the synaptic cleft [44, 63, 80, 81]. Both actin- and microtubule-depolymerizing agents significantly disturbed the synaptic clustering of Gag and Env molecules in the effector cell reducing the cell-cell viral spreading [42, 48].

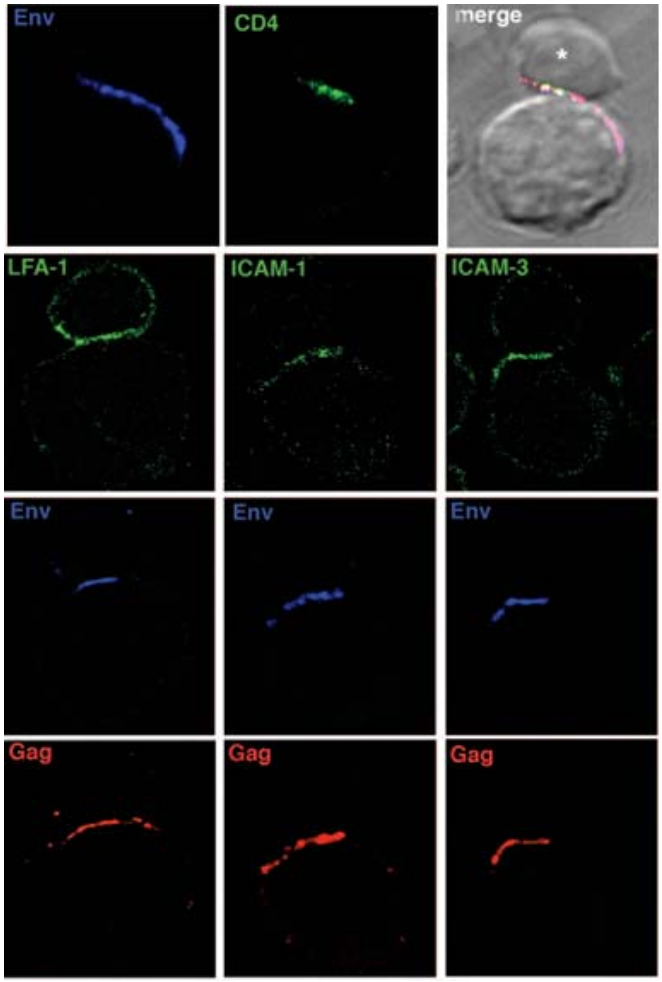


Figure 7. Co-localization of CD4, Gag, LFA-1, ICAM-1, and ICAM-3 at the VS. Illustration from [82].

However, actin may be cleared out from the central region of the VS, a strategy proposed to facilitate viral entry into the target cell [83]. The MTOC polarization towards the synaptic cleft mediates the polarized secretion of granules during IS assembly [84, 85] a feature that retroviruses might hijack for their own spread across VS. As described for IS where TCR engagement mediates ZAP-70 signaling, during VS, ZAP-70 was also essential for efficient Gag clustering, MTOC relocalization and viral transmission even though VS formation occurs independently of TCR recruitment [86].

The HIV capture by target cells following contact with HIV-infected cells has been documented by antibody-mediated detection of viral products [45], direct visualization of fluorescent HIV Gag products [87], and electron microscopy analysis [3, 63]. However, some aspects of the mechanisms of virus transfer deserve further clarification such as the intrinsic infectivity of the viral material transferred to target cells. For this, the role of coreceptor during the early events of the VS formation has recently been compromised. As observed for cell-free HIV infection, coreceptor engagement was required for HIV to infect target cells [13-15] but the role of coreceptor during cell-to-cell HIV transmission presents a more complex scenario. *In vitro*, when uninfected CD4 T cells express the appropriate coreceptor, the X4 or R5, Env-expressing cells bind to coreceptor and the principal Env-gp41 functions are switched on: cell-to-cell fusion and syncytia formation, target cell death and/or

infection (Figure 8). Several studies have been aimed at characterizing the role of coreceptor engagement at the VS but many controversies have risen between different groups on whether coreceptor engagement at the VS is required to observe an efficient cell-cell HIV spread and hence will be discussed in more detail in this thesis.

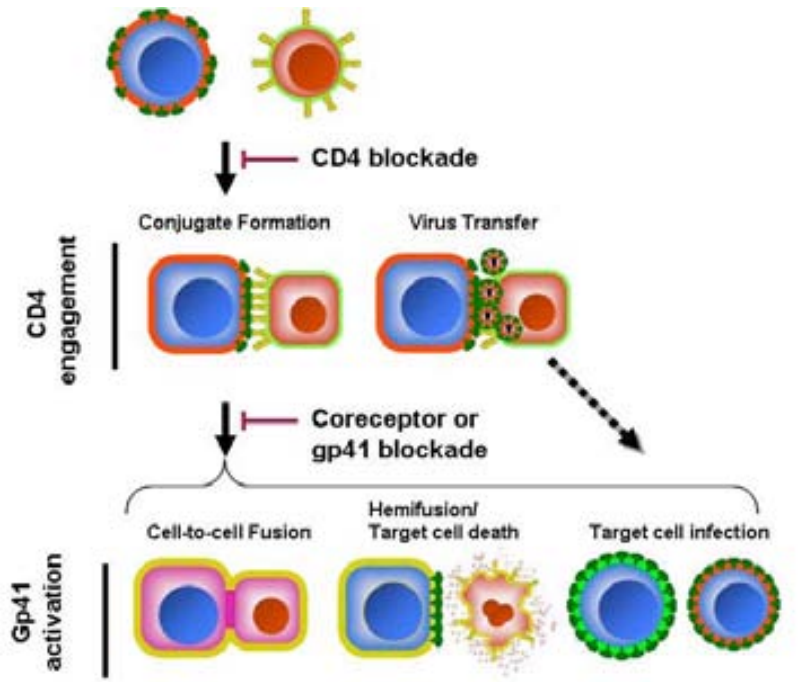


Figure 8. Diagram of the events occurring at the HIV T cell-T cell VS. Env-CD4 interaction allows VS formation and transfer of viral particles to target cells. Upon coreceptor engagement, gp41 activation may lead to cell-cell fusion and subsequent syncytia formation, hemifusion-mediated cell death and/or target cell infection.

2.2.4 The role of adhesion molecules in VS

The active contribution of LFA-1/ICAM-1 interaction to HIV spread has been described during free virus infection of CD4 T cells [34, 88-90], DC-T cell HIV transmission [91-93], and in cocultures of placental cells with T lymphocytes [94]. HIV incorporates ICAM-1, placed on the surface of the cell membrane, during the budding process and these ICAM-1-bearing viruses increase the infectivity rate of HIV binding to target cells. Either in cell-free or DC-T cell viral transmission, LFA-1 increases viral infectivity and directs infection towards the $CD45RO^{+}$ memory CD4 T cell subset [39]. Lack of the expression of the CD18 subunit of LFA-1 leads to Leukocyte Adhesion Deficiency type-1 (LAD-1), a rare disorder where the CD11/CD18 heterodimers can not pair intracellularly, and hence LFA-1 is not expressed at the cell surface of leukocytes from LAD-1 patients [92]. In a study of DC-T cell HIV transmission using lymphocytes from LAD patients, HIV transfer was inhibited in the absence of LFA-1 expression [92]. On the other hand, tetraspanins are described as membrane organizers, selectively clustering proteins into microdomains leading to cell-cell fusion, cell adhesion, and cell motility [95] (and reviewed in [96]) that are recruited at the sites of HIV budding during VSs [97, 98]. The overexpression of the tetraspanins CD63, CD9 and CD81 on effector cells *in vitro* reduced cell-free infectivity as well as cell-cell HIV transmission and fusion [95, 99]. These phenomena would correlate with the fact that HIV mediates downregulation of these molecules

as a beneficial mechanism to ensure its own spread [95, 99]. However, the involvement of adhesion molecules in the transmission of HIV between infected and uninfected CD4 T cells is poorly defined. Although LFA-1 may modulate the formation of cellular conjugates and synaptic structures [82], a clear correlation between LFA-1 expression and T cell-T cell HIV transmission has not been described and will be studied in more detail throughout this thesis.

2.2.5 Transport mechanisms at the VS: endocytosis, nanotubes, filopodia and trogocytosis

The classical model of HIV infection of CD4 T lymphocytes establishes that Env-mediated fusion at the plasma membrane is required for HIV core entry into the cytoplasm, often leading to a productive infection [100] (Figure 9A). However, HIV can also enter target cells using the endocytic route [101-103] (Figure 9B). The acidic condition within endosomes facilitates conformational changes in the Env glycoprotein that permit fusion of the viral and endosomal membranes in a pH independent fashion [7, 104, 105]. Yet several studies have shown that HIV particles can be captured into endocytic compartments; electron microscopy data from an early description [81] to the recent report by Chen *et al* [78], described polarized Gag or Gag-GFP budding at the VS and endosomal vesicles containing HIV particles in target cells. Furthermore, biochemical data showed how transferred materials were directed towards trypsin resistant compartments [104].

Likewise, video-microscopy experiments illustrated the vesicular transfer of large amounts of Gag, sporadically involving entire synaptic buttons [78]. All in all, these data supports the endocytic HIV transmission mechanism, a process that requires the extracellular but no the intracellular moiety of CD4 [22].

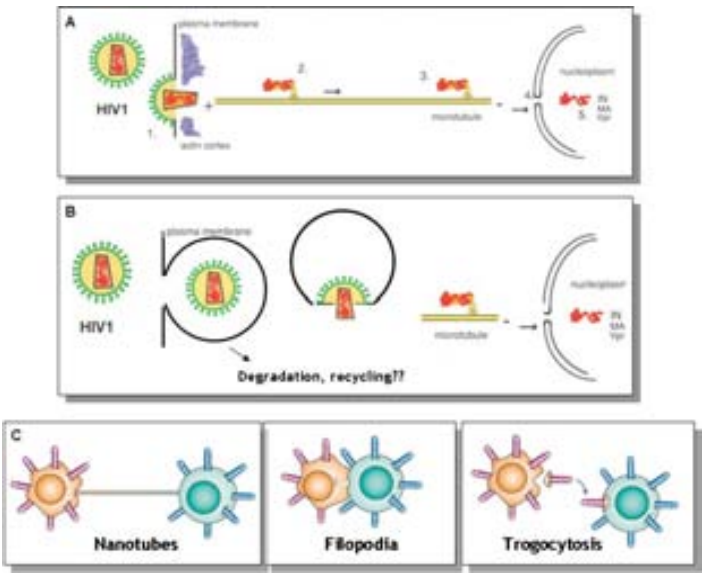


Figure 9. Mechanisms of HIV transfer between immune cells. **A.** HIV particles fuse at the membrane level and travel through the microtubules to reach the nucleus and establish the infection. **B.** HIV particles are incorporated into endosomal compartments, which may go on for degradation, recycling or rather fuse at the endosomal membrane level where HIV would have a short distance to the nucleus. **C.** Membrane nanotubes or filopodia perhaps derived from membrane fusion or membrane bridges at the site of intercellular contact, could facilitate protein/HIV transfer between distal cells. Trogocytosis, which drags entire membrane patches between cells in contact, could occur throughout the formation of membrane bridges. Illustration adapted from [106]

A part from the mechanism of endocytosis, alternative cell-to-cell virus transfer mechanisms that are not mediated by large contact zones (such as the VS) have been described. These include *nanotubes*, identified as long cytoplasmatic bridges that enable directed communication between cells. Nanotubes have been reported in T lymphocytes and have been proposed for cell-to-cell HIV transfer [46, 107] suggesting that HIV hijacks nanotube communication capacity in order to spread [108, 109] (Figure 9C). Another mode of retroviral transfer involves the establishment of *filopodial bridges*, also referred to as cytonemes, between infected and target cells (Figure 9C) [110, 111]. In this case, the receptor-expressing target cell extended filopodia towards the infected cell, in which they were anchored by partial endocytosis [111]. *In vitro* culture fibroblasts produce abundant filopodia [112] whereas primary lymphocytes do not, so it is questionable the relevance of this mechanism of HIV spread *in vivo*. Both filopodia and nanotubes might allow transfer to distant cells by viral particles presumably surfing along the bridged structures, as observed not only with retroviruses, but also with multiple viral species, like herpesvirus, papillomavirus, and vaccinia virus [113-117]. Nanotubes differ from filopodia in that they are 10-fold longer than filopodia and electron microscopic analysis suggested that the point of intercellular nanotube contact represents a small scale VS where HIV transfer is mediated by Env binding to its receptors [46].

Trogocytosis (from the ancient Greek *trogo*, meaning "gnaw" or "nibble") was described as the transfer of plasma membrane fragments from a presenting cell to a lymphocyte [118] and has been documented in T, B and natural killer cells both *in vitro* and *in vivo* [106]. Trogocytosis can be distinguished from other mechanisms of intercellular transfer as being a process that allows rapid transfer of intact cell-surface proteins accumulated at the IS [106]. Moreover, the easy monitoring of trogocytosis can be achieved with the flow cytometry technology using a wide variety of membrane probes that can be tracked easily [119]. The best-studied example of protein transfer that occurs by trogocytosis is that of peptide–MHC complexes transferred from APCs to T cells [106, 120, 121] yet a recent study has determined the preferential molecules transferred by trogocytosis *in vitro* [122] . Trogocytosis, though, is not merely an uprooting of cell-surface receptors but also involves transfer of whole membrane patches [106] (Figure 9C). The directionality of the membrane transfer has recently been studied by the group of Dr Hudrisier which showed that depending on the cell type used, trogocytosis could occur in a unidirectional or bidirectional direction [123]. Not only its molecular mechanism, but also the functionality of trogocytosis has yielded rather interest in the field of cell-to-cell HIV spread. Trogocytsosis has been able to modulate the initiation, duration and termination of the immune responses as well as endowing cells with uncharacteristic phenotypes not normally associated with that particular cell type [106]. This latter observation could lead to the transfer of viral receptors to cells that

are not normally expressing them and hence render those cells susceptible to viral infection [106, 124]. Therefore, trogocytosis has been studied in this thesis as a possible mechanisms allowing HIV transfer across VS.

Nonetheless, it remains to be elucidated if all these different transport mechanisms that take place at the VS may have implications during HIV transmission, particularly leading to the infection of target cells.

3 Cell-to-cell HIV spread and the generation of latently infected cells

In HIV infected individuals, viral production is a dynamic process involving continuous rounds of infection of CD4 T lymphocytes with rapid turnover of virus producing cells that have a half-life of 1–2 days [125]. The highly active antiretroviral treatment (HAART) for HIV infected patients consists of a combination of antiretroviral drugs targeting different steps of the HIV cycle: entry, reverse transcription, integration and maturation. This treatment leads to a decrease in viral load in plasma of infected individuals, which rebounds if the treatment is interrupted. The decay curves of plasma viremia following HAART have shown that after an initial fast decay that wipes out the majority of circulating viruses in 1–2 weeks, plasma virus declines at a lower rate. The half-life of this

compartment was estimated to be 1–4 weeks, but the nature of the cellular reservoir responsible for the second phase in the decay curve is still unclear. These cells could be macrophages, which are less sensitive to the cytopathic effect of HIV infection, CD4 T lymphocytes not fully activated, which carry the integrated provirus in a non-replicative state until the activation process is complete or dendritic cells (DCs) not permissive for HIV infection but carrying the virus trapped into intracellular vesicular compartments. We were aimed to evaluate the impact on the establishment of HIV reservoirs into CD4 T lymphocytes after T cell-T cell VS engagement.

3.1 Target cells for HIV infection: activation not a prerequisite.

Activated CD4 T cells are particularly susceptible to infection and render them permissible for HIV to gain access and replicate (Figure 10). When HIV gets this activation environment, it can easily replicate and as a consequence CD4 T cells are severely depleted a feature that is observed in the course of an HIV infection. More than 90% of the virus present in plasma originates from this activated cellular source in which their lifespan is truncated by HIV infection [5, 126].

However, a more controversial scenario has lately shown up; cells under a quiescent state isolated from blood of infected patients on HAART have appeared latently infected *in vivo* [126]. Besides, other non-diving cells such as neurons or macrophages also supported HIV infection [127]. The cell cycle of these infected resting CD4 T cells is arrested in a G0/G1a state and no activation markers are found in the surface of these latently infected cells (Figure 10). Even though quiescent CD4 T cells can get infected, they do not produce viral proteins and are thus difficult to distinguish from uninfected cells. This and the fact that the frequency of latently infected CD4 T cells *in vivo* is low (1-10 cells per 10^6 CD4 T cells) [128] makes it difficult to study HIV latent infection *in vivo*.

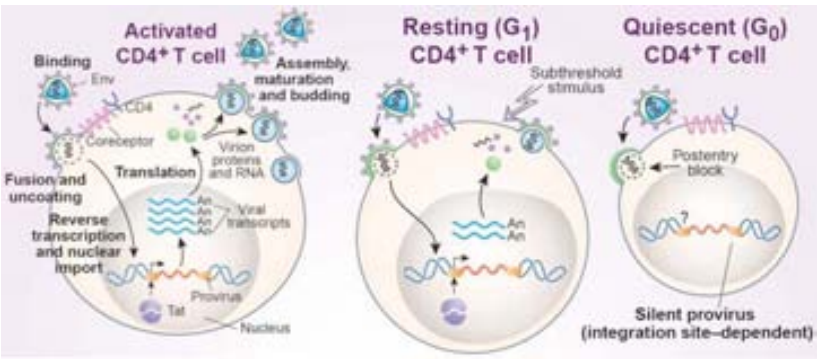


Figure 10.

Figure 10. Viral replication patterns in different host cells. In activated CD4 T cells, viral replication is efficient and cytopathic. Subthreshold stimuli (such as cytokines) may be sufficient for the cell to enter G1 but are insufficient for complete progression and activation of the cell cycle. Such cells support infection, but the extent of viral gene expression and virus production is reduced relative to activated cells. In a quiescent (G0) infected CD4 T cell, proviruses are considered to be transcriptionally silent. Illustration adapted from [5]

Many efforts are being done in finding an *in vitro* model that faithfully represents the key characteristics of latently infected cells and the same microenvironment found *in vivo*. Cell lines are not the best representative model of the resting phenotype since they are immortalized cells that continuously divide. The use of primary cells might not be a good tool either due to their short-term survival in *in vitro* culture. Of note, a recent model to generate long-lived primary qCD4 T cells *in vitro* has been proposed in which qCD4 T cells can get infected and do not require a full cellular activation phenotype [129]. The general features required to obtain latently infected cells *in vitro* are:

1. *Isolation of a pure population of resting CD4 T cells*. The most commonly way used to isolate qCD4 T cells is to determine the cell-surface markers CD25, CD69 and HLA-DR that indicate early, intermediate and late stages of T-cell activation, respectively. Moreover, flow cytometry separation by morphological parameters can distinguish resting T cells for their small and uniformed size.

2. *Detection of integrated HIV DNA into the host cell genome*. When HIV enters the nucleus of the target cell might remain predominantly as unintegrated DNA [130, 131] or rather recombine with the host cell genome. Several assays have been developed to detect the integrated provirus in resting CD4 T cells by a quantitative real-time PCR (qPCR) approach using a set of primers that bind to the host genome encoded Alu and HIV sequences [132-135].

3. *Ensure that viral replication occurs after the adequate stimulation*. Latently infected qCD4 T cells would raise HIV productive infection when they acquire an activated phenotype. However, post-stimulation of qCD4 T cells latently infected *in vitro* did not reach the efficient replication capacity observed in previously stimulated cells [136].

3.2 Molecular mechanisms of HIV latency

There is a diverse opinion about the different points where productive HIV infection of qCD4 T cells is halted. The main discussion relies on the integration of the HIV provirus into the host cell genome. Thus, two general forms of viral latency have been observed and can be segregated based on whether or not the virus integrates into the host cell genome: preintegration and postintegration latency [137, 138]:

3.2.1 Pre-integration latency

This theory relies on the partial or complete block of the viral life cycle at steps prior to the integration of the virus into the host genome. This block could result in several steps of the HIV life cycle prior to integration:

Uncoating: TRIM5α, a cellular restriction factor of retroviral replication, was responsible for the inhibition of the release of virions into the cytoplasm of cells from a type of monkey [139].
Reverse transcription: an incomplete RT was observed as a result of a reduced dNTP pool which might explain the slow kinetics of reverse transcription in resting CD4 T cells [139, 140] . APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like editing complex 3) is a member of a family of cytidine deaminases enzymes from the host cell that inhibit retroviral replication. APOBEC3G introduces mutations in the viral DNA that is produced by reverse transcription, and these mutations inhibit further DNA replication by mechanisms that are not fully defined. However, activation of T cells converts cellular APOBEC3G into an inactive complex [141]. Moreover, the HIV infectivity factor Vif binds to APOBEC3G and promotes its degradation by cellular proteases, thus Vif is critical for *in vivo* HIV replication [142].

3.2.2 *Integration*

When HIV enters the nucleus of the target cell it can either integrate or remain as unintegrated DNA (Figure 11). The latter represents the largest fraction of HIV DNA present in the nucleus of infected cells [143]. The first evidence of non-integrated DNA *in vivo* was reported by Pang and colleagues [144]. It mainly accounts for linear DNA, the 1-LTR and the 2-LTR episomal DNA. The ends of the unintegrated linear DNA may be joined to form a 2-LTR circle, which can also recombine between the two LTRs yielding circles with a single LTR (1-LTR) [133, 145] (Figure 11). The unintegrated linear HIV DNA has the ends easily accessible for the host cell enzymes a fact that might explain the circularization as a repair process to reduce the forthcoming cellular danger signals [146]. Remarkably, the 2-LTR form has been widely utilized by several authors as an indicator of newly HIV infected cells [145-147]. The reason for this supposition is mainly based on the unstable feature of this episomal DNA, which also decreases in concentration as a result of cell division. The 2-LTR form was easily monitored by using a set of primers that amplify across the LTR circle junction [148].

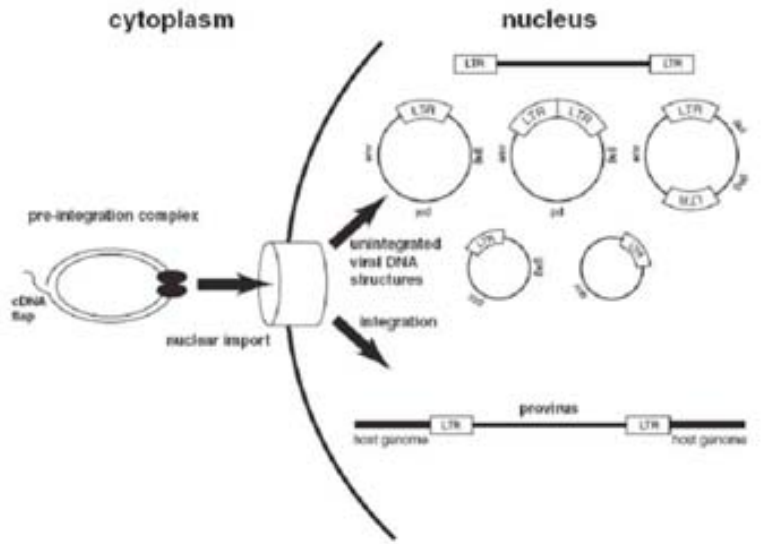


Figure 11. Schematic description of the generation of the multiple HIV DNA forms. The pre integration complex (PIC) contains the viral dsDNA with its triple-helical region (flap) and the virus-derived proteins including integrase. After passing through the nuclear pore, the linear HIV DNA may be integrated into the host chromosome to generate the provirus. Alternatively, it may stay non-integrated linear or may undergo circularization to one of the various forms (1-LTR and 2-LTR) [149].

3.2.3 *Post-integration latency*

In the steps beyond integration, the HIV provirus that has been able to integrate, failed to effectively express its genome and remained reversibly silenced unless appropriate stimulation. This latent state is exceptionally stable and is limited only by the lifespan of the infected cell and its progeny. Several aspects may contribute to the transcriptional silencing of the integrated HIV provirus:

The sites of integration. Apparently, HIV integration seemed to be a random process but in an analysis of purified resting CD4 T cells from patients on HAART, HIV provirus was mainly detected within introns of actively expressed genes. Thus, it suggested that the presence of integrated proviruses within actively transcribed host genes is not sufficient for the productive infection of resting CD4 T cells [150]. Besides, a more recent study has compared the HIV integration sites in resting and activated CD4 T cells and have found no differences although a high frequency of incorrect integration and abortive circular viral forms were observed in unstimulated cells [151].

The cellular transcription factors. There are two key host transcription factors; nuclear factor of activated T cells (NFAT) and the nuclear factor of activated B cells (NF B) that reside in the cytoplasm of resting T cells and are recruited to the nucleus upon cellular activation. Their function is to transcribe several genes that are

important for T cell function as well as the HIV genes to produce viral particles. Mainly they act by interacting to the HIV LTR where the transcription of the integrated provirus starts. The differential location of NFAT and NF B in resting and activated CD4 T cells (cytoplasm and nucleus, respectively) may hamper the transcription of the HIV genome in resting lymphocytes contributing to the establishment of HIV latency.

The regulatory HIV proteins. There are two major regulatory proteins encoded by the viral genome involved in the replication process; Tat and Rev. Tat is produced early in the replication cycle and plays a primary role in the expression of viral transcripts by binding to the tat activation region (TAR) present in the LTR region. Tat preferentially recognizes RNA, and acts at the level of elongation [152]. The Rev protein is involved in the expression of genes encoded by unspliced mRNA. Incomplete spliced mRNAs are retained into the nucleus to allow splicing to occur and subsequent export of the transcripts into the cytoplasm and translation of functional proteins. Rev function by interacting to the cis-acting Rev Response Element (RRE) found on unspliced viral RNAs and targets them for export to the cytoplasm overriding the cellular machinery in charge of processing the unspliced RNAs. Even though the early HIV proteins Tat and Rev have been detected in HIV infected resting CD4 T cells, no viral production occurred [153]. It remains unclear why these two regulatory proteins alone do not increase virus gene expression to levels sufficient for virus production in

resting CD4 T cells. A post-transcriptional block in the export of the multiply spliced RNA may well explain the latency of infected CD4 T cells [154].

Thus, the search of new combinations of HIV or host factors that might be involved in the establishment of HIV latency is needed. A multifactorial process seems to be responsible for hampering the post-transcriptional activity in resting T cells. Overcoming this step might render the cell infectious and several reports agree that transcription of the integrated HIV provirus is the matter of debate to finally eradicate HIV infection [153, 154].

3.3 Cell-to-cell HIV transmission supports infection of qCD4 T cells

A common immune response is observed when CD4 T cells recognize their cognate antigen by the classical MHC-class II presentation during DC-T cell contact and undergo a clonal expansion of activated effector cells to respond against the antigen. The majority of these cells would die by activation-induced cell death mechanisms but eventually some cells could revert back to a quiescent state and persist as memory cells, allowing rapid responses to future challenges with the same antigen [155]. DCs are the most potent antigen-presenting cells of the immune system and yet they play a pivotal role in the establishment and dissemination of the HIV infection *in vivo* by promoting a high viral transfer to

target CD4 T cells [156]. Not only the high transfer of virus but also the stimulation that DCs confer into their target cells, enabled HIV infection of qCD4 T cells upon coculture with autologous DCs [157]. Therefore, the question remains unknown whether HIV-infected qCD4 T cells found *in vivo* are due to previously stimulated T cells that have survived and revert into a resting state [126] or rather infected during its resting phenotype. In HIV-infected individuals, a small percentage (0,01%) of resting CD4 T cells isolated from blood contained integrated proviruses, and the majority presented a memory phenotype which suggested that prior activation enabled the infection [158, 159].

On the contrary, a recent report suggested that a cell does not have to be overtly activated to support viral infection since subtle stimulatory signals delivered to CD4 T cells may promote entry into a G1 state of the cell cycle sufficient for the virus to enter the nucleus of the target quiescent cell without the expression of activation markers [160]. It has been proposed that Env-signaling through coreceptor interaction may allow HIV to gain access to downstream cellular components, which can then serve as effective tools to break cellular barriers and facilitate pathogenesis [161, 162], a feature that was not observed by pseudotyping the HIV virion with the vesicular stomatitis virus glycoprotein (VSV-G) [161, 163]. Other concomitant results showed HIV RNA production in CD45RA CD4 T cells of HIV infected individuals [164] and HIV infected lymphoid tissues [165]. The expression of the CD45RA surface marker

indicates a naïve subset of T cells observed in non-activated cells suggesting that prior activation was not a perquisite to observe viral production into this CD4 T cell subset [165].

A recent communication from Dr. Chen, B.K and colleagues showed an increase in the HIV proviral DNA found in CD4 T cells after cell-to-cell contact as compared to cell-free viral infections measured by fluorescence in situ hybridization (FISH) [166]. This observation may support the idea that HIV mediated cell-to-cell contacts between infected and uninfected CD4 T cells increases the infectious rate of CD4 T cells without the requirement of previous stimulation and has also been explored in this thesis.

HYPOTHESES & OBJECTIVES

It has been widely documented that HIV can spread *in vitro* and *in vivo* across the cells of the immune system. This mode of transmission is highly efficient as compared to the "classical" route of entry by cell free virus binding to a permissive host cell. This efficiency relies on the formation of structures termed "virological synapses". The contribution of the adhesion molecules on free virus infection and DC-mediated cell-to-cell HIV transmission has been previously described although their role in the transmission between infected and uninfected CD4 T cells is still a matter of debate.

Different mechanisms of cell-to-cell HIV transmission have been already described between cells of the immune system though the exact mechanism for T cell-T cell HIV spread has been less studied. Regarding the role of coreceptor, essential for HIV entry and infection, two different virological synapse models' have been proposed; dependent and/or independent of coreceptor engagement. Besides, other routes need to be explored such as the contribution of trogocytosis in cell-to-cell HIV transmission.

Although cellular activation is necessary to achieve a productive HIV infection, qCD4 T cells can get latently infected in the absence of any stimulation. The effectiveness of cell-to-cell HIV transmission may enhance the infection rate of qCD4 T cells enabling the establishment of viral reservoirs. Identifying the latently infected cells *in vivo* is a major point in the final eradication of the disease.

Thus the objectives set for this study can be divided as early, inner or late events occurring during cell-to-cell HIV transmission between infected and uninfected CD4 T cells:

Early events

1. To define the morphology of the VS, especially the interaction of the Env glycoprotein to its receptor CD4 and the adhesion molecules; LFA-1 binding to its main ligand ICAM-1 and their role during T cell-T cell HIV transmission.

Inner events

2. To evaluate the impact and outcomes on the formation of T cell-T cell VS dependent and/or independent of coreceptor.

3. To determine, in particular, the effect of trogocytosis during the physical pass of HIV particles across the VS.

Late events

4. To unravel the activated phenotype of quiescent CD4+ T cells latently infected as a result of T cell-T cell HIV transmission.

MATERIALS AND METHODS

1. Cells

1.1 Primary cells

1.1.1 Obtained of PBMCs

Buffy coats (i.e. the fraction of blood enriched in white blood cells and platelets) from healthy donors were obtained from the Banc de Sang i Teixits (BST, Hospital Vall d'Hebrón, Barcelona, Spain) and approved by the Ethical Committee of the center. The buffy coat underwent a standard Ficoll-Hypaque (Lymphoprep™) density gradient purification to isolate the desired fraction of peripheral blood mononuclear cells (PBMCs). After centrifugation a layer of clear fluid on the top (the plasma), a layer of red fluid containing most of the red blood cells on the bottom, and a thin layer in between, making up less than 1% of the total volume of the blood sample, which contains the desired cell population of PBMCs were detected. Then, the "cloud" of mononuclear cells was collected and washed twice with PBS 1% and resuspended in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% of heat inactivated fetal bovine serum (FBS). Finally, cells were counted using a micro-beads system of a known concentration (Perfect Count Mycrospheres™, Cytognos) following manufacturer's instructions.

1.1.2. Purification of T cell subsets.

PBMCs were used immediately for further purification of T cell subsets. Whole CD4 T cells, memory CD4 T cells ($CD45RO^+$) and naïve CD4 T cells ($CD45RA^+$) were purified by negative immunomagnetic selection using the appropriate cocktails of antibodies (Miltenyi Biotec SL). Purity of isolated populations (>95%) was assessed by flow cytometry after CD4, CD45RO and CD45RA staining with the appropriated antibodies. All cells were cultured in RPMI1640 medium supplemented with 10% heat inactivated FBS in the absence of any other stimuli.

The approach used to obtain *quiescent CD4 T cells* was to remove activated cells that stained positive for the activation markers CD25, CD69 and HLA-DR out from purified CD4 T cells. First, CD4 T cells were labeled with 1μL/10^6 cells of the anti-CD25, anti-CD69 and anti-DR antibodies labeled with PE. Then qCD4 T cells were purified by negative selection using a PE selection Kit (Stem Cell Technology). The protocol involved a first step using an anti-PE antibody that binds to the PE-conjugated CD25, CD69, and DR antibodies on the surface of activated cells. Secondly, cells were labeled with dextran-coated magnetic nanoparticles that recognize both the dextran and the PE molecules on the PE-conjugated antibody. These nanoparticles attached labeled cells on the column magnet surface so that unlabeled qCD4 T cells were negatively isolated. This protocol allowed removing almost all activated CD4 T cells as it was assessed by flow cytometry after the purification (<5% cells remained positive for the CD25, CD69, DR antigens) (Figure 12).

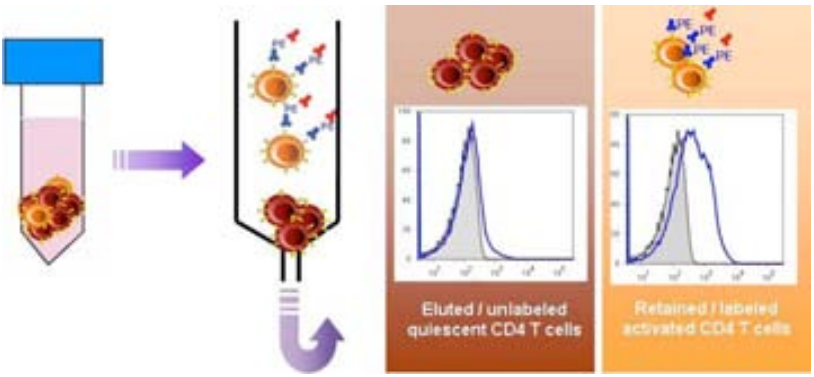


Figure 12. Schematic representation of the qCD4 T cell purification. Activated CD4 T cells (orange) were surface bound with PE-conjugated mAbs against CD25, CD69 and DR. Addition of the anti-PE antibody conjugated to magnetic beads allowed for the retention of the activated cells inside the column and elution of the non-labeled quiescent CD4 T cells (Fraction 1). At the end of the purification, activated CD4 T cells that were magnetically bound to the column were vigorously shacked and eluted in the bound fraction. Histograms show the multiple CD25/CD69/DR staining for each eluted fraction of CD4 T cells (blue lines). Unstained cells were used as a negative control (grey histograms).

1.1.3 Stimulation of primary cells.

For the stimulation of PBMCs or purified CD4 T cells, 4μg/mL of phytohaemagglutinin (PHA) and 6U/mL of interleukine-2 (IL-2) were added and cells incubated for 3 days at 37ºC and 5%CO_2 at a final concentration of 2 x10^6 cells/mL. Then, cells were washed to remove the PHA and cultured again in RPMI-1640 supplemented with 10%FBS maintaining the concentration of IL-2 in culture.

1.2 Cell lines

1.2.1 Cells in suspension:

The human T-lymphoblastoid cell line MOLT obtained from an acute lymphoblastic leukemia was employed for the study of cell-to-cell HIV transmission events. This cell line endogenously expresses the CXCR4 receptor and was transfected with a plasmid coding for the CCR5 gene. The uninfected MOLT cell line has been chronically infected with an X4 (NL4-3) or an R5 (BaL) ($MOLT_{NL4-3}$, and $MOLT_{BAL}$) virus in the laboratory by Dr. Julià Blanco. The immortalized Jurkat cell line of T lymphocyte origin was used as target cells for HIV transmission expressing a $CD4^+CXCR4^+$ phenotype and obtained trough the NIH AIDS Research and Reference Reagent Program. As these cells grow in suspension, they were cultured with the RPMI1460 medium supplemented with 10% FBS at 37ºC and 5%CO_2. Routinely, cells were passed twice a week leaving an approximately concentration of $2{,}5x10^5$ cells/mL.

1.2.2 Adherent cells:

The embryonic kidney cell line 293T ($CD4^-$, $CXCR4^-$) was obtained from the NIH AIDS Research and Reference Reagent Program and were appropriate to work with in transfection experiments. The 293T cell line was transfected by Dr Anne Aucher with a vector coding for the fusion protein CD4-GFP, the CD4 alone or an empty vector (kindly provided by Dr G.Gaibelet, INSERMU563, Hôpital Purpan, Toulouse, France). The TZM-bl ($CXCR4^+$) cell line was

isolated from a clone of the HeLa cell line stably transfected with CD4 and CCR5 (previously designated JC35-bl). Besides, it had integrated copies of the luciferase and ß-galactosidase genes under the control of the HIV promoter being highly sensitive to infection with diverse HIV isolates. TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program. All adherent cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% FBS at 37ºC and 5%CO_2. Cells were passed twice a week at a dilution 1/10 when they reached the maximal flask-surface confluence. The mouse mastocytoma cell line P815 was kindly provided by the group of Dr D.Hudrisier (CNRS; IPBS ;Institut de Pharmacologie et de Biologie Structurale, Toulouse). This cell line naturally expresses FcγRII/III receptors and it was stably transfected to express the human CD4 receptor fused or not to the green fluorescent protein GFP (P815 CD4-GFP and P815 CD4, respectively). These cells grew adherent until they reach the maximal surface confluence when they switch to grow in suspension. They were cultured at 37ºC and 5%CO_2 with RPMI1640 medium supplemented with 5% FBS and passed three times a week.

2. Viral stocks

2.1 Obtained in PBMCs

PBMCs were cultured in the presence of 4μg/mL PHA and 6U/mL IL-2 for 2 days. Stimulated T cells were then incubated with an NL4-3 stock virus (previously obtained in PBMCs) for 4 hours at a multiplicity of infection (M.O.I) of 1 (defined as the number of virus used to infect one target cell) followed by two washes with PBS and incubated again in the presence of 10U/mL of IL-2 for the next 3 days to preserve them under survival conditions. At 3 days post-infection, the supernatant of the infected PBMCs cells was harvested and filtered using a 0.3-4 μM filter to remove the possible remaining cells. The viral stock was employed to infect primary CD4 T cells used in experiments of cell-to-cell HIV transfer.

2.2 Obtained from transfection

Subconfluent 293T cells were seeded in a 6-well plate and left over-night cultured in DMEM-10% FBS medium. Cells were transfected the following day when they had reached over 50-80% confluence with an HIV NL4-3 expression plasmid lacking a functional Env (pNL4-3.Luc.R-.E-; NIH AIDS Reagent Program) together with an Env expression plasmid pCDNA3-NL4-3 at a ratio 1:2 using the Calphos transfection system. The CAp24 protein is encoded in the ΔEnv plasmid that produces the CAp24 polyprotein used as a

marker to track HIV transfer by intracellular staining. Cotransfections generated pseudo-viruses that allow for one round of HIV replication into the target cell since they only carry the DNA vector from the ΔEnv plasmid (Figure 13). The lack of Env expression generates HIV particles devoided of the Env glycoprotein that will be used as negative controls of infection. Due to the lack of expression of adhesion molecules on 293T cells as determined by immunophenotyping, a third vector coding for ICAM-1 or ICAM-3 (kindly provided by Dr M.Juan, Hospital Clinic de Barcelona) was added into the cotransfections to test the effect of these adhesion molecules on HIV transfer (Figure 13). The cell-surface expression of these adhesion molecules to evaluate the efficiency of transfection was measured by flow cytometry at 24 hours post-transfection as described above using the RM3A5 (anti-ICAM-1) and the 140.11 (anti-ICAM-3) antibodies.

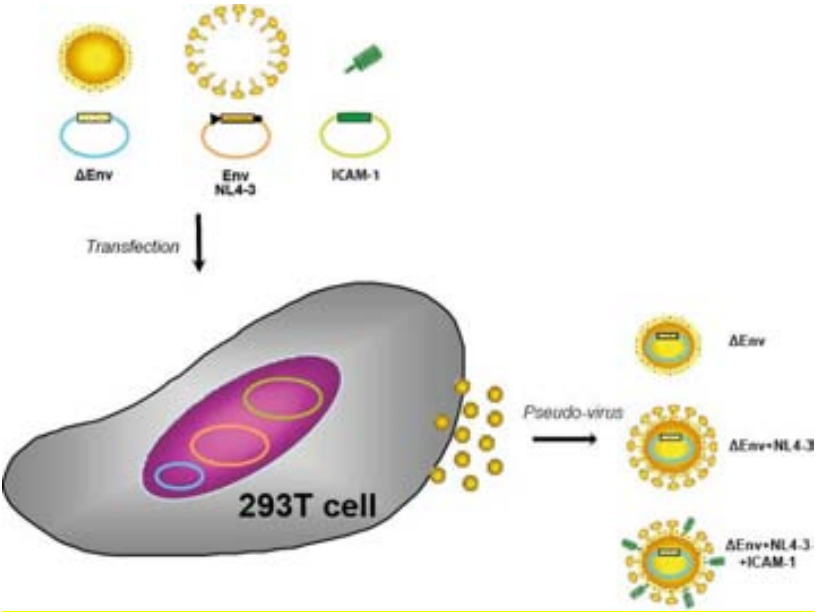


Figure 13. Schematic representation on the generation of pseudoviruses. 293T cells were transfected with a ΔEnv alone or in combination with an Env NL4-3 plasmid. Addition of a third plasmid coding for ICAM-1 or –3 was used to obtain pseudovirus expressing either ICAM-1 or -3 on their surface.

2.3 Titration and quantification of HIV cell-free virus

The infectivity of each supernatant carrying cell-free virus was tested following a luciferase-based experiment. TZM-bl cells were seeded in a 96-well plate at $1x10^5$ cells/well. Addition of 37.5μg DEAE-dextran/ml was used to help enhance the infection of the cells. Then serial dilutions 1/10 of the supernatants were added to

wells sequentially and the plate incubated for 48 hours at 37ºC, 5%CO_2. TZM-bl cells contain a luciferase gene controlled under the HIV promoter. Thus, when HIV infects TZM-bl cells, it activates transcription of the luciferase gene and its activity was analyzed by luminometry using the Britelite Reagent (Britelite Luminescence Reporter Gene Assay System, Perkin Elmer Life Sciences) following manufacturer's instructions. Calculation of the 50% Tissue Culture Infective Dose (TCID50) was determined following the Montefiori's protocol [167]. This value accounts for the amount of a pathogenic agent that will produce pathological changes in 50% of cell cultures inoculated and is expressed as $TCID_{50}$/mL.

The concentration of viral particles present in the supernatants of infected/transfected cells was calculated using a commercial sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) of the HIV CAp24 antigen. To calculate the concentration of HIV particles in each sample, a standard curve using a known CAp24 concentration was performed in parallel. Absorbencies obtained for each viral sample were extrapolated to the standard equation and represented as nanograms (ng) of CAp24/mL.

3. Reagents

3.1 Anti-HIV compounds

The monoclonal antibody against CD4 (Leu3A clone) that specifically blocks the interaction with the Env glycoprotein was purchased from BD Bioscience. The monoclonal antibody against the CD4 binding site of the gp120 Env subunit (IgGb12) was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The synthetic CXCR4 antagonist bicyclam JM-2987 (hydrobromide salt of AMD-3100) was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH [168]. The CCR5 antagonist TAK779 was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH [169]. The gp-41 derived peptide C34 covering the sequence 628-661 in the second extracellular helical region of gp-41 Env subunit was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The HIV integrase inhibitor Raltegravir (commercially available as Isentress) was obtained from Merck Sharp&Dohme. The HIV retrotranscritpion inhibitor AZT (3 -Azido-3 -deoxythymidine) was purchased from Sigma-Aldrich.

3.2 Antibodies

Molecule name	Antibody Clone	Cluster	Isotype	Source
β2 integrin lymphocyte function-associated antigen-1 (LFA-1, αLβ2)	68.5a5	CD18	IgG2a	Dr. R Vilella, Hospital Clinic, Barcelona
	R7.1	CD11a	IgG1	Ebioscience , San Diego, USA
	24	CD11+CD18 activation epitope	IgG1	Abcam, Oxford, UK
Intracellular adhesion molecule-3 (ICAM-3)	101.1D2	CD50 Domain 1	IgG2a	Dr. R Vilella, Hospital Clinic, Barcelona
	CBR-IC3/1		IgG1	Axxora, Switzerland
	140.11	CD50 Domain 2	IgG2a	Dr. R Vilella, Hospital Clinic, Barcelona
	140.11 Fab *		-	Fab preparation kit, Thermo scientific
	186.2g9		IgG2b	Dr. R Vilella, Hospital Clinic, Barcelona
	152.2d11	CD50 Domain 1&2	IgG2a	Dr. R Vilella, Hospital Clinic, Barcelona
Intracellular adhesion molecule-1(ICAM-1)	Recombinant soluble ICAM-1**	CD54	-	R&D Systems
	Rm3a5		IgG1	Dr. R Vilella, Hospital Clinic, Barcelona
Basigin, a type I integral membrane glycoprotein	HIM6	CD147	IgG1, κ	BD, Bioscience
Intergrine β-1	18/CD29	CD29	IgG1	BD Bioscience

Table 1. Antibodies against adhesion molecules used for inhibition assays. After titration by flow cytometry, the above-described antibodies were used at saturating concentrations in coculture for cell-to-cell HIV transmission assays. Besides, functionality of the anti-LFA-1 and anti-ICAM1 antibodies has been previously assessed [170].

* The Fab fragment of the anti-ICAM-3 antibody 140.11 was obtained by digestion of 10 mg of whole IgG using a papain-based commercial kit (Fab Preparation Kit, Thermo Scientific) following manufacturer's instructions. Eluted samples were collected in several fractions and the absorbance analyzed, meaning the concentration of Fab protein eluted in each fraction. The Fab fraction

was observed in fractions 4, 5 and 6 whereas the Fc unwanted fraction appeared from fraction 16 onwards (Figure 14). After digestion, Fab was purified using a Protein A column that yielded 90% pure Fab preparations, as assessed by SDS-PAGE. Main contaminants were Fc fragments, without detectable IgG (Figure 14).

** A recombinant soluble peptide encoding the 455 amino acid residue form of human ICAM-1 minus the transmembrane and cytoplasmic domains was purchased from R&D Systems and used for inhibition assays

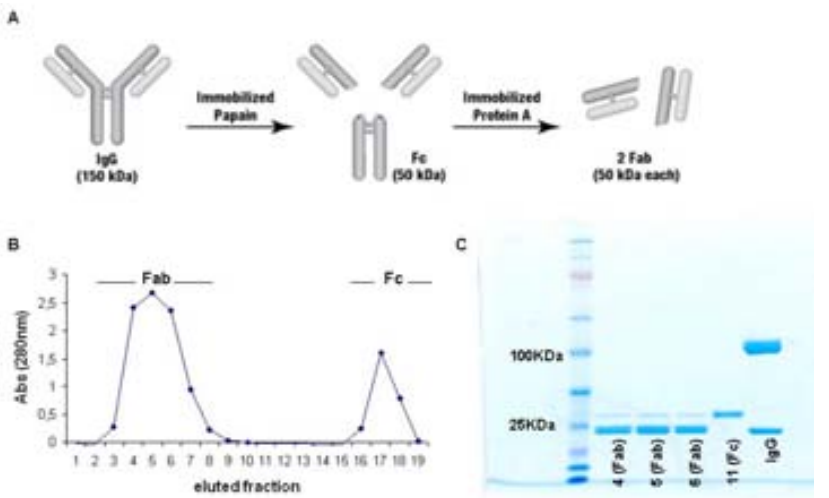


Figure 14

Figure 14. Digestion of the anti-ICAM-3 mAb 140.11. **A**. Schematic representation of Fab digestion. **B.** Representation of the absorbance (260/280) obtained from the various fractions collected after Fab digestion and subsequent purification. **C.** SDS-PAGE gel was performed to measure the purity of the obtained fractions. Fab fractions that presented the higher absorbance values (4, 5 and 6) were loaded. The Fc fraction eluted after the step 16 and the whole IgG were also loaded to determine the remaining contaminants.

MOLECULE	CLONE	FLUOROPHORE	SOURCE
CD4v4	L120	FITC	BD Bioscience
CD45 RO	UCHL1	APC	
CD45 RA	HI100	APC	BD Bioscience
Rabbit anti-Human IgG Rabbit F(ab')2	Polyclonal	FITC	Dako Cytomation
Goat anti-mouse IgG	polyclonal	FITC	BD Bioscience
CD25	M-A251	PE	BD Bioscience
CD69	L78	PE	BD Bioscience
HLA-DR	TU36	PE	BD Bioscience
ZAP-70	pY292	PE	BD Bioscience
Lck (p56)	pY505	Alexa Fluor 647	BD Bioscience
CD3	HIT3a	-	BD Pharmingen
CD28	CD28.2	-	BD Pharmingen
FcγRII/III	2.4G2	-	Dr Hudrisier, IPBS, Toulouse

Table 2. Antibodies used for immunophenotyping (white boxes) or blocking assays (grey boxes).

3.3 Other compounds

The cell trackers DDAO (Cell Trace™ Far Red), CMRA (Cell Tracker™ Orange) and CMFDA (Cell Tracker™ Green, 5-chloromethyl fluorescein diacetate) and the lipophylic probes DiO (3,3'-dioctadecyloxacarbocyanine perchlorate, Molecular Probes) and DiI (1,1' - Dioctadecyl - 3,3,3',3' tetramethyl- indocarbocyanine iodide, Molecular probes) were purchased from Invitrogen and used to stain cells in several experiments. The DNA extraction kit QIAamp DNA Blood kit was purchased from Qiagen. For 293T cell transfections, the Calphos transfection system was purchased from Clonetech and used following manufacturer's instructions. To intracellularly stain the CAp24 antigen, the Fix&Perm kit purchased from Caltag was used. The indirect ELISA of the Gag HIV antigen purchased from Innogenetics was used to determine the concentration of viral particles in cell supernatants. The plasmids ICAM-1 and ICAM-3 were kindly provided by Dr. M.Juan. The Britelite Luminescence Reporter Gene Assay System was purchased from Perkin Elmer, Life Sciences and used for titration of viral stocks. The Pierce® Fab Micro Preparation Kit that uses the papain enzyme to obtain the Fab region of an antibody was purchased from Piercenet, Thermo Scientific. The Perfect Count Mycrospheres™ purchased from Cytognos, were used to count cells by flow cytometry. The PE Selection kit was purchased from Stem Cell. The CD4, T CD45RO and T CD45RA cell isolation kits by negative selection were purchased from Miltenyi Biotech.

4. Immunophenotyping by flow cytometry

The expression of CD4, Env, ICAM-1,ICAM-3, LFA-1, the active form of LFA-1 was determined in effector MOLT cells (uninfected or HIV infected), 293 T cells, PBMCs (uninfected or HIV infected) and purified CD4 T cells. $2x10^5$ cells were incubated for 20 minutes in the presence of 1µg/mL of the corresponding antibodies in a 96 V-bottomed plate. Then, cells were washed with PBS and incubated with 1µg/mL of FITC-labeled goat anti-mouse antibody for 20 minutes. The expression of HIV Env was determined by using a pool of serums from HIV infected patients (dilution 1/10). After washing out unbound antibodies, Env expression was revealed using a FITC-coupled goat anti-human antibody. Cells labeled with the FITC-conjugated secondary antibody were used as negative control. Finally, cells were washed twice with PBS and immediately fixed with 150µL of formaldehyde 1% before analysis by flow cytometry in a LSR II cytometer equipped with a plate loader (BD, Bioscience).

Analysis was made into the different populations identified by forward and site scatter values (FCS and SSC, respectively). For quantitative evaluation of cell surface expression, Relative Fluorescence Intensity (RFI) values were represented as the Mean Fluorescence Intensity (MFI) of positive samples divided by the corresponding negative controls of staining.

5. Cell-to-cell HIV transmission: Coculture models

5.1 MOLT cells as effector cells

$MOLT_{NL4-3}$, and $MOLT_{BAL}$ cells were cocultured with primary CD4 T cells at a ratio 1:1. Uninfected MOLT cells were used as a negative control of HIV infection. Cocultures were incubated at 37ºC and 5%CO_2 to analyze cellular conjugates, transfer of the HIV CAp24 antigen and membrane lipids at 2 hours and cell death, syncytia formation and proviral DNA synthesis at 24 hours.

On the other hand, uninfected and infected MOLT cells were cocultured with the MT-4 cell line at a ratio 1:1. As MT-4 present a similar morphology than the MOLT cells based on the FCS and SSC values, MT-4 cells were labeled previous to the coculture. To do so, the cell tracker DDAO was loaded into cells by adding 1µL of the previously DMSO resuspended tracker to 1mL of cells resuspended in RPMI1640 for 30 minutes at 37ºC and 5%CO_2, followed by two PBS-washes to eliminate the excess of tracker. These reagents pass freely through cell membranes, but once inside the cell, are transformed into cell impermeable reaction products and hence are not transferred to adjacent cells. Labeled MT-4 cells were cocultured and transfer of the CAp24 antigen and membrane-associated lipids was analyzed into DDAO-gated MT-4 cells by flow cytometry at 2 and 24 hours.

5.2 Infected primary cells as effector cells

PBMCs from healthy donors were infected with a NL4-3 virus stock as described previously. In the following days, the expression of the CAp24 antigen, CD3, CD4, and CD8 was monitored by flow cytometry as described [18]. Only $CD3^+/CD8^-/CD4^-/CAp24^+$ cells were considered productively infected due to the complete downregulation of CD4 from the surface of HIV infected cells. These cells were purified when the percentage of $CD3^+/CD8^-/CD4^-/CAp24^+$ reached 3% or above using a ratio 1:1 of the cocktails of antibodies used to purify CD4 and CD8 T cells by negative selection in order to obtain the desired eluted fraction (CD4 and CD8 negative). Purity of the isolated population was assessed by flow cytometry after CD4 and CAp24 staining and compared to the retained fraction (CAp24 negative). Enriched infected cells were usually > 50% CAp24 bright (Figure 15).

Infected primary CD4 T cells were then cocultured with uninfected primary CD4 T cells at a ratio 2:1. In this case, as both cells present the same morphological parameters when assessed by flow cytometry, the uninfected CD4 T cell population was previously labeled with the intracellular cell tracker CMFDA (as previously described). Cocultures were incubated for 24 hours at 37°C and 5% CO_2 and HIV transfer analyzed at 24 hours.

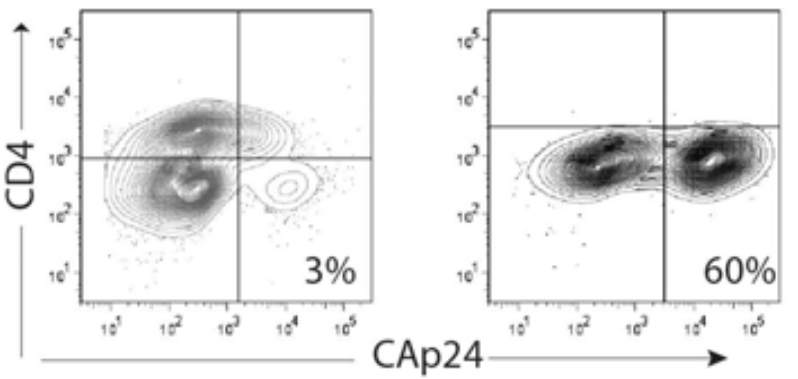


Figure 15. Purification of infected primary CD4 T cells. At 3 days post-infection, PBMCs were analyzed for their CAp24 content. Double staining analysis of surface CD4 receptor and intracellular CAp24 antigen determined the population of CD4 productively infected that have downregulated the CD4 cell-surface expression. When the percentage of PBMCs $CAp24^{+}CD4^{-}$ reached >3%, cells were purified with the CD4 and CD8 negative selection cocktails and purity of the isolated population assessed by again double staining with CD4 and intracellular CAp24. A purity >50% was accepted.

5.3 HIV-transfected 293 T cells as effector cells

HIV-transfected 293 T cells (See viral stocks section) were cocultured at 24 hours post transfection with purified primary CD4 T cells at a ratio 2:1. The backbone plasmid lacking the functional Env was used alone as a control of potential Env-independent nonspecific transfer (i.e. MOCK). Addition of the ICAM-1 or -3 plasmids to the transfection was used to monitor the effect of these adhesion molecules on cell-to-cell HIV transfer. Cocultures were left 24 hours at 37ºC and 5%CO_2 prior to the HIV transfer analysis.

6. Quantification of cellular conjugates

To analyze the contacts established between uninfected or infected MOLT cells and primary target cells, purified CD4 T cells were first labeled with 1μL/mL of the cell tracker CMRA as previously described. To analyze the selectivity of contacts towards a subset of CD4 T cells, purified memory CD4 T cells ($CD45RO^+$) and purified naïve CD4 T cells ($CD45RA^+$) were differently labeled with the cell trackers CMRA and CMFDA respectively. Then labeled cells were cocultured with infected MOLT cells at a ratio 1:1 ($2x10^5$ primary CD4 T cells or $1x10^5$ CD45RO + $1x10^5$ CD45RA cells for mixed cocultures of memory and naïve target CD4 T cells) for 2 hours at 37° with or without shaking conditions on a 96 well flat-bottom plate. Inhibitors tested were: the anti-CD4 mAb Leu3A (0,25 μg/ml), the fusion inhibitor peptide C34 (5 g/ml), the CXCR4 antagonist JM-2987 and the CCR5 antagonist TAK779 (both used at 10 go/ml). The following mAbs R7.1 (LFA1), mab24 (activated LFA-1) RM3A5 (ICAM-1) and 140.11 (ICAM-3) were used at 10 μg/ml. Following the incubation time, 50 μL/well of formaldehyde 5% in PBS were added to the cultures without perturbing cellular conjugates, which were analyzed 15 minutes later in a LSRII flow cytometer equipped with a plate loader. All the events with similar morphology to MOLT cells and at the same time being positive for the cell tracker label (CMRA or CMFDA) were considered to be stable cellular conjugates between an effector MOLT cell and a target primary CD4 T cell. Percentage of cellular contacts was calculated as follows: [conjugates (conjugates +CD4 free cells)] x100.

7. Evaluation of the CAp24 antigen transfer

The amount of HIV particles transferred to target cells was quantified by intracellular staining of the HIV CAp24 antigen using the Fix&Perm kit. Initially, 100μL of the coculture present in 96-well plates were transferred to a new V-bottomed 96-well plate to perform the staining protocol in a plate. Immediately cells were centrifuged and the pellet fixed in 25 μL of the solution A for 15 minutes at RT. After, cells were washed with 200μL of PBS and resuspened in 25μL of the PE-labeled monoclonal CAp24 KC57 antibody diluted 1/50 in the permeabilization solution B and incubated for 20 minutes longer at RT in the dark. Finally, cells were washed twice with PBS to remove the excess of the bubbly solution B. Cells were finally fixed with 150μL of formaldehid 1% in PBS and analyzed 15 minutes later with the flow cytomter LSRII provided with a plate reader. Quantification of the CAp24 antigen transfer was evaluated into the target cell population as the percentage of events that stained positive for the CAp24 antigen. In cocultures of MOLT cells or HIV-transfected 293T with primary CD4 T cells, the initial criterion used to identify the target population was the different morphological parameters of effector cells compared to primary CD4 T cells. On the other cases a cell tracker was employed as described above to distinguish between the two counterparts of the coculture. Two hours of coculture were sufficient to observe transfer of HIV particles from the chronically infected MOLT cells, although 24 hours were necessary for cocultures using HIV-

transfected 293 T cells or infected primary cells as effector cells. In parallel, monitorization of the percentage of effector MOLT cells or 293T cells for their CAp24 content was also assessed (Figure 16). In the experiments performed to determine selectivity of transfer towards a CD4 T cell subset, cells were surface stained with an APC-labeled anti-CD45RO antibody for 15 min and washed with PBS prior to the fixation. CAp24 transfer was then analyzed in the gated CD45RO positive cells (memory) and CD45RO negative cells (naïve).

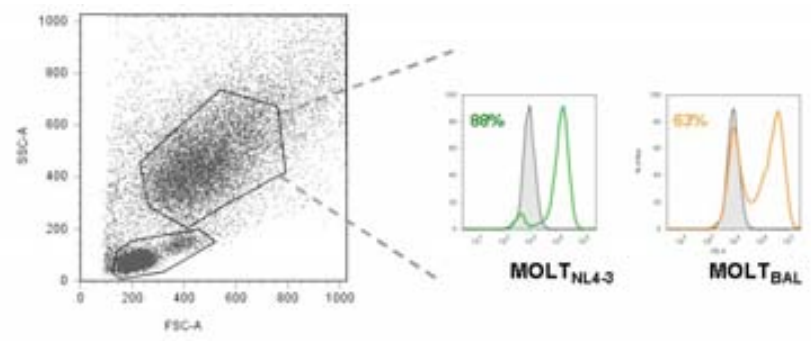


Figure 16. Analysis of the CAp24 antigen content in the chronically infected MOLT cell line. Cocultures of primary CD4 T cells with MOLT$_{NL4-3}$, and MOLT$_{BAL}$ cells were performed. FSC and SSC parameters allow for the identification of both cellular populations (left dot plot). Gating on the MOLT cells, analysis of CAp24 antigen content was measured by intracellular staining. Uninfected MOLT cells were used as negative controls of CAp24 staining (grey) into which the NL4-3 (green) and BaL (orange) staining was over-layed. The percentages of either NL4-3 or BaL CAp24 positive cells are introduced in each correspondent histogram.

7.1 Trypsin treatment

In order to determine the percentage of viral particles able to reach the cytoplasm of target cell, the protease trypsin was used to cleave the cell surface-bound HIV particles. For cocultures of primary cells as target cells it has been previously described that trypsin treatment does not affect the extent of viral particles transferred [104]. However, its effect for the MT-4 cell line in cocultures with infected MOLT cells was further analyzed. For trypsin treatment, cocultures of MOLT cells with primary CD4 or MT-4 cells were washed with versene and treated for 10 min at RT with 0.25 % trypsin solution. Addition of FBS stopped trypsin action. Cells were then washed with PBS and intracellular stained for the CAp24 antigen as indicated above. Quantification of HIV transfer into target cells was assessed by the ratio of MFI of infected versus uninfected samples.

8. Evaluation of lipid transfer

Transfer of membrane was also assessed in cocultures of chronically infected MOLT cells either with primary CD4 or MT-4 cells. Cocultures were incubated 24 hours at 37ºC and 5%CO_2. MOLT cells were previously labeled with 34 M of the green lipophylic probe DiO that diffuses laterally to uniformly stain the entire cell membrane, incubated for 15 minutes at 37ºC and 5%CO_2 and extensively washed with PBS to remove the unbound probe. The

MT-4 cells were stained with the cell tracker DDAO as previously described. Analysis of membrane transfer to primary CD4 T cells or DDAO-labeled MT-4 cells was performed by flow cytometry in samples previously fixed with formaldehyde 1% in PBS. Addition of the anti-CD4 mAb Leu3A (0,25 μg/mL) and the fusion peptide C34 (5 μg/mL) were used as inhibitors of membrane lipid transfer.

9. Cell death and syncytia formation

Cell death was measured in cocultures of infected MOLT cells with primary CD4 T cells at 24 hours. Cells were analyzed for their morphological parameters (i.e. FSC and SSC values) by flow cytometry. Total CD4 T cells were gated separately from MOLT cells. Inside the CD4 T cell gate, the events with the lower FCS and SSC values were gated as death CD4 T cells whereas the remaining events were considered as living CD4 T cells.

Syncytia formation was evaluated in cocultures of infected MOLT cells and primary CD4 T cells at 24 hours. Multiple cell fusion was evaluated by simply observing the cocultures at the microscope. Giant cells with a balloon-like structure were considered dead syncytia. Uninfected MOLT cells were used as negative controls of HIV-mediated syncytia formation. Besides, C34 (5 μg/mL) was added as a fusion and consequent cell death inhibitor.

10. Analysis of HIV transmission by qPCR

For the analysis of HIV transmission by quantitative PCR (qPCR), cocultures of $5x10^5$ CD4T cells with $5x10^5$ chronically infected MOLT cells were performed in a 48-well plate. Cocultures were conducted in the absence or the presence of the following inhibitors; C34 (5μg/mL), Leu3A (0,25μg/mL) and 10μg/mL of anti-ICAM-1 (RM3A5), anti-ICAM-3 (140.11 and its Fab fragment), anti-CD11a (R7.1) and anti-CD18 (68.5a5). At 24 hours, DNA from the cocultures was extracted (QIAamp DNA Blood kit, Qiagen) and stored at -20ºC. Cocultures stopped at time zero were used as a negative control of transmission. Each sample was amplified in triplicate using a Taqman universal PCR Master Mix, and primers and probes for HIV and CCR5. All results were normalized to the number of cells in each case by amplifying the endogenous gene (CCR5). Primers used to amplify total HIV DNA were F: 5´ GACGCAGGACTCGGC TTG3´ and R: 5´ ACTGACGCTCTCGCACCC3´ and the probe: 5´ CGGCGACTGGT GAGTACGCCAAA 3´. Primers used to amplify the CCR5 gene: F: 5′ TCATTACACC TGCAGCTCTCATTT 3′ and R: 5′ ACACCG AAGCAGAGTTTTTAGGAT 3′ and the labeled probe 5′ CTGGTCCTGCCGCTG CTTGTCA 3′. Reactions (40 cycles of 15s at 95ºC for melting and 1 min at 60ºC for annealing/extending) were conducted in an ABI7000 Sequence Detection System (Applied Biosystems). Relative proviral DNA synthesis was calculated using the $2^{-\Delta\Delta CT}$ method [171]. $\Delta\Delta CT = (CT_{HIV} - CT_{CCR5})_{Test} - (CT_{HIV} - CT_{CCR5})_{Control}$, where CT is the fractional cycle number that reaches a fixed threshold.

11. Trogocytosis: Coculture models

Trogocytosis as described in the introduction is a process of antigen transfer between cells. In order to test whether trogocytosis may have an impact during cell-to-cell HIV transmission, different coculture models were performed in which trogocytosis was analyzed in both HIV transmission directions.

11.1 Re-directed trogocytosis assay

Capture of plasma membrane fragments from mouse FcR-expressing cells (P815) expressing or not the CD4-GFP fusion protein was measured into CD8 T cells (Figure 17A). Prior to the coculture, P815 cells were irradiated at 60 grays (Gy) to stop cell division and primary CD8 T cells were incubated with 10 g/mL of the anti-CD3 and anti-CD28 mAbs for 15 minutes at 4°C to coat the cell surface with mouse IgGs. The re-directed trogocytosis principle is explained by the interaction of the mouse IgG coated on the surface of CD8 T cells with the mouse FcR receptor expressed on the surface of P815 cells. Experimentally, irradiated P815 cells were cocultured with mab-coated CD8 T cells at a ratio 1:5, for 1 hour at 37°C. Addition of the blocking anti-FcγRII/III mAb 2.4G2 or cocultures with P815 cells lacking the CD4-GFP construct were used as negative controls of re-directed trogocytosis. After 1 hour, CD8 T cells were analyzed by flow cytometry using an anti-CD8 mAb to gate on the cells of interest and tracking the percentage of GFP expressing cells as those that have been able to capture CD4-GFP upon trogocytosis.

11.2 Trogocytosis from uninfected to infected cells

11.2.1 CD4 transfer:

Cocultures of $5x10^5$ 293T cells expressing the CD4-GFP fusion protein with $5x10^5$ $MOLT_{NL4-3}$, or $MOLT_{BAL}$ cells were seeded on a 96-well plate. MOLT cells were labeled with DDAO as described before in order to differentiate them from the 293T cell line. 293T cells carrying an empty pCDNA3 vector or uninfected MOLT cells were used as controls of Env-CD4 independent trogocytosis (Figure 17B). After 2 hours of coculture, cellular contacts were disrupted by vigorously shaking and 100µL of the coculture transferred into a new 96-well plate. Cells were fixed by adding 100µL of formaldehid 1% in PBS and incubated for 15 min prior to the flow cytometry analysis. Trogocytosis was assessed into the DDAO-labeled MOLT cells by measuring their GFP content. Inhibitors tested were C34 (5 µg/mL) and Leu3A (0,25 µg/mL) as well as the neutralizing antibody IgGb12 (30 µg/mL).

11.2.2 Membrane-associated lipid transfer:

Cocultures of $5x10^4$ chronically infected MOLT cells with $5x10^4$ 293T cells expressing or not the CD4-GFP protein were incubated for 2 hours at 37ºC and 5%CO_2. On the one hand, transfer of membrane lipids was assessed as previously described using this time the membrane lipophylic probe DiI (Figure 17B). On the other hand, 293T cells were labeled with the cell tracker CMRA as described above in order to stain the cytoplasm of the cell. Analysis of

membrane/cytoplasm transfer to DDAO-labeled MOLT cells was performed by flow cytometry in samples previously fixed with formaldehyde 1% in PBS. The anti-CD4 mAb Leu3A (0,25 μg/mL) was used as inhibitor of the Env-CD4 interaction.

11.3 Trogocytosis from infected to uninfected cells

11.3.1 Gag-dependent transfer:

To explore the mechanism of trogocytosis as a candidate to explain viral transfer at the VS, intracellular staining of the HIV antigen was assessed using 293 T cells either transfected with an Env-expressing plasmid (pCDNA3-NL4-3) as a WT or the fusion defective mutant 41.2 cloned in a pCDNA3 in combination with a ΔEnv plasmid at a ratio 2:1 (Figure 17C). After 24 hours $1x10^5$ transfected 293T cells were cocultured with $5x10^4$ primary CD4 T cells in a 96-well plate and incubated for 24 hours. Cells were then stained for the intracellular CAp24 antigen (as described above). The anti-CD4 mAb Leu3A (0,25 μg/mL) and the fusion peptide C34 (5 μg/mL) were used as inhibitors of Env-CD4 interaction and fusion, respectively.

11.3.2 Gag- independent transfer:

In this case, 293T cells transfected alone with the Env-WT or the fusion defective mutant 41.2 were used as effector cells. Transfer in this case was independent of gag-driven HIV particles as the cell-surface Env directs by itself the formation of the cellular conjugates

in the absence of particle release (Figure 17C). Twenty-four hours post-transfection, 293T cells were membrane-labeled with 1 µL/mL of DiI (as described above). Then, $1x10^5$ DiI-labeled 293T cells were cultured with $5x10^4$ CD4 T cells for 24 hours at 37°C and 5%CO_2 and transfer of membranes analyzed by flow cytometry into gated primary CD4 T cells.

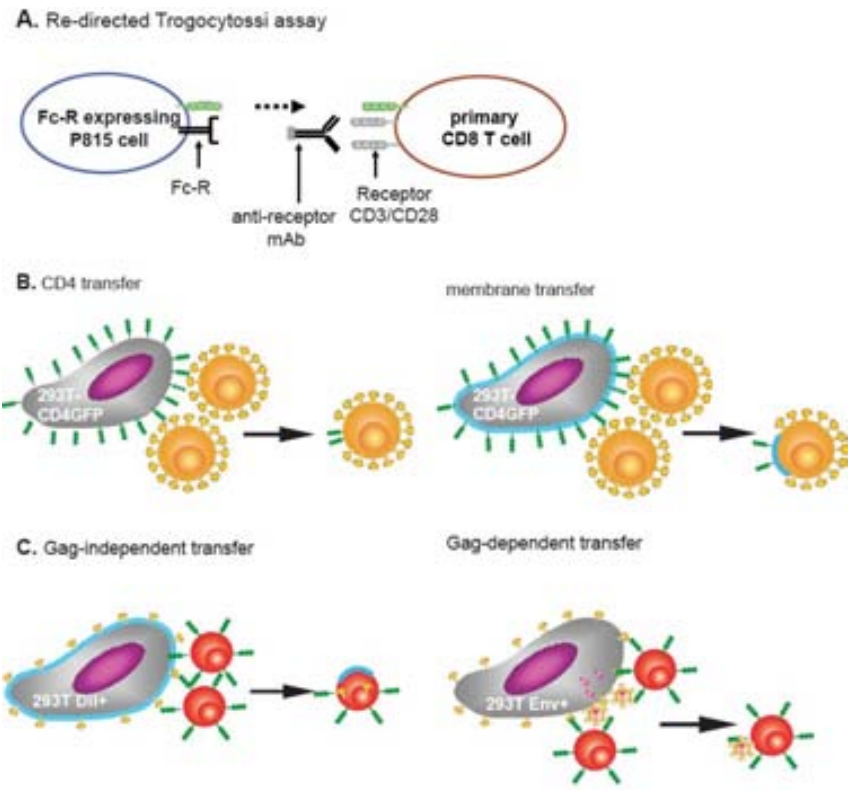


Figure 17

Figure 17. Schematic representation of the different trogocytosis systems. A. The principles of redirected trogocytosis between Fc-R expressing cells and primary CD8 T cells previously coated with mAbs (adaptation from [172]). The Fc-R receptor recognizes the Fc fragment of the antibodies bound on T cells and induces the cross-link that allow for trogocytosis of the CD4-GFP fusion protein into primary CD8 T cells. **B.** Transfer from uninfected to infected cells; 293T cells stably transfected with the fusion protein CD4-GFP, transfer the CD4-GFP to chronically infected MOLT cells (CD4 transfer). 293T CD4-GFP cells are labeled with the membrane probe DiI and transfer of membrane patches are observed into MOLT infected cells (membrane transfer). **C.** Transfer from infected to uninfected cells; HIV tranfected 293T cells are cocultured with primary CD4 T cells and transfer of CAp24 antigen assessed by intracellular staining (CAp24 dependent-transfer). 293 T cells transfected with an Env-containing plasmid are membrane-labeled and transfer of membrane patches analyzed in cocultured primary CD4 T cells (CAp24 independent transfer).

12. Evaluation of different forms of HIV DNA by qPCR

Cocultures of $5x10^5$ primary CD4+T cells with $5x10^5$ chronically infected MOLT cells were either seeded in 48-well plates or in a 96-well plate using a transwell system to compare cell-to-cell transmission versus free-viral infection respectively. MOLT cells were placed on the top of the membrane and primary CD4 T cells at the bottom. The membrane had a porous size ranging from 0.4-3μm sufficient to exclusively allow MOLT-released viruses to cross to the bottom compartment and infect target cells but not the cells. Addition of C34 (5 μg/mL) and Raltegravir (1μM) were used as controls of HIV entry and integration respectively. Cocultures stopped at time zero were used as negative controls of cell-to-cell

transmission. After 24 h, DNA was extracted using the QIAamp DNA Blood kit, and the different forms of viral DNA quantified by qPCR. The Total (1-LTR) and the 2-LTR DNA amplifications were normalized relative to the CCR5 gene copy numbers as determined from the chromosomal fraction. Amplifications of total, 2-LTR-episomal HIV DNA and CCR5 were performed in duplicates. The PCR reaction was conducted under the same conditions as mentioned above. To amplify the 1-LTR form primers used were contained inside the HIV LTR region (R: 5´TTA AGC CTC AAT AAA GCT TGC C 3´ and U5: 5´GTT CGG GCG CCA CTG CTA GA 3´) and the probe was labeled with 6-FAM on the 5´end and TAMRA on the 3´end (5´CCA GAG TCA CAC AAC AGA GGG GCA 3´). To amplify the 2-LTR form the following primers were utilized: 5´CTA ACT AGG GAA CCC ACT GCT 3´ and 5´GTA GTT CTG CCA ATC AGG GAA G 3´ at the U5 and U3 position, respectively and the probe labeled with 6-FAM on the 5´end and TAMRA on the 3´end (5´AGC CTC AAT AAA GCT TGC CTT GAG TGC 3´). These primers bind to the junction between the 2-LTR thus excluding all other forms of viral DNA. In the case of total HIV DNA, primers employed amplify the 1-LTR sequences so that all forms of DNA are included in the analysis (episomal, lineal and integrated). All primers and probes were purchased from Applied Biosystems. In order to quantify HIV infection, an HIV infected cell line that harbors a plasmid containing the sequence of either the 1-LTR or the 2-LTR and the CCR5 gene was used to generate a standard curve. Values from all samples were extrapolated into this standard curve to obtain the number of HIV copies/cell [148].

13. Analysis of productive HIV infection

To monitor HIV productive infection of target cells by flow cytometry analysis, 293T cells were transfected with a backbone vector that carries a form of enhanced green fluorescent protein (EGFP) in the Env open reading frame (pNL4-3-deltaE-EGFP) co-transfected with the pCDNA3 NL4-3 Env-expressing plasmid. Efficiency of transfection was measured 24 hours later by flow cytometry as the percentage of 293T cells positive for GFP. 293T cells transfected alone with the backbone were used as negative control of productive infection. Transfected 293 T cells were cocultured either with PHA-stimulated CD4+ T cells, quiescent CD4+ T cells or DDAO-labeled MT-4 cells for 24 hours at a ratio 2:1. The fusion peptide C34 (1μg/mL) and the integrase inhibitor Raltegravir (1μM) were added as controls of HIV entry and integration, respectively. HIV productive infection was assessed by flow cytometry in target cells (primary CD4 or MT-4) by analyzing the number of GFP positive cells.

14. Intracellular expression of the phosphorylated p56Lck and ZAP-70 forms

Primary purified CD4 T cells were left overnight in RPMI medium supplemented with 10% FBS since the Ficoll treatment may have activated the cells. The following day, primary CD4 T cells were stimulated using the following antibodies commonly used for activation studies: a cocktail of anti-CD3 and anti-CD28 (10µg/mL) monoclonal mouse IgG antibodies were incubated at 4ºC for 15 minutes. Unbound antibody was washed with PBS followed by the incubation for 15 minutes at 4ºC with an anti-mouse IgG antibody to allow the crosslink and hence activation of CD4 T cells. Immediately after incubation, cells were placed at 37ºC for 5 minutes longer to induce phosphorylation of intracellular signaling proteins p56Lck and ZAP-70. These conditions were used as positive controls of phosphorylation. In parallel, primary CD4 T cells were cocultured with $MOLT_{NL4-3}$, cells at a ratio 1:1 and incubated for 30 minutes at 37ºC, 5%CO_2 at a high concentration in order to maximize cellular contacts. Previously activated cells and the cocultures were then fixed (Cytofix Buffer, BD Bioscience) and permeablized (BD Phosflow Perm/Wash Buffer) using the BD PhosFlow kit for human PBMCs following the manufacturer's instruction. Cells were stained with 20µL of the anti-p56Lck and anti-ZAP-70 antibodies against the phosphorylated forms labeled with PE and APC respectively in separate tubes and analyzed by flow cytometry. The population of primary CD4 T cells was identified by forward and site scatter

values and for their content of the phosphorylated forms of p56Lck and ZAP-70. Unstimulated qCD4 T cells were used as negative controls of phosphorylation of p56Lck and ZAP-70 whereas the IgG cross-linked CD4 T cells used as the maximal phosphorylation levels that these cells could reach.

15. Proliferation assay

In order to observe cell proliferation qCD4 T cells were labeled with the cell tracker CMFDA as previously described. Proliferation of the labeled cells was monitored by flow cytometry analysis. With each round of cell division the relative fluorescent intensity (RFI) decreases by half. Coculture of $MOLT_{NL4-3}$ cells with CMFDA-labeled qCD4 T cells were performed at a ratio 1:1 and the CMFDA fluorescence intensities were followed at day 3 post coculture. Stimulated CD4 T cells (as described before) were used as a positive control of cellular proliferation. Cells were fixed with formaldehid 1% in PBS and assessed by flow cytometry. To quantify the number of events undergoing division, analysis was conduced by gating the qCD4 T cell population and measuring the decrease in FITC fluorescence intensity. Values were calculated as percentage of cells that have undergone cellular division.

DNA of cells was labeled using the propidium iodide (IP) probe in order to measure the cell division state. To do so, the same cocultures used for CMFDA staining were divided in two to stain

both CMFDA and IP separately. IP staining was performed adding 1:500 of dye (3μM) in staining buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl2, 0.5 mM MgCl2). Cocultures were incubated for 15 minutes at RT and analyzed by flow cytometry provided with the plate reader.

16. Statistical analysis

Statistic analysis was performed using two-sided Student's *t* test. Bonferroni correction was performed when indicated. P values less than 0.05/n were considered to indicate statistical significance (n=number of comparisons).

RESULTS

Chapter I: *Cell-to-cell HIV Transfer vs. Transmission*

Although cell-to-cell HIV spread was defined in the early 90's, in the last five years, several groups have underscored the relevance of this mode of HIV spread between productively infected and uninfected CD4 T cells by defining the term VS. However, there is a controversy among the different groups when analyzing data on cell-to-cell HIV spread. The disagreement is mainly set in the role of coreceptor during the formation of VSs. By studying the functional role of coreceptor in different cellular models (primary cells and cell lines); we suggest that primary cells are highly sensitive to the physical pass of viral particles across the synapses, a coreceptor-independent phenomenon that we call "HIV transfer". Once viral particles are transferred, they can infect target cells by a coreceptor-dependent mechanism that fits with the classical meaning of "HIV transmission" and that is much more efficient in cell lines. The differences in the ability of primary CD4 T cells and cell lines to support HIV transfer and transmission explain most of the reported controversial data and should be taken into account when interpreting the results. Thus, we propose to uniformly use the nomenclature transfer and transmission accordingly.

In summary, HIV particles would be transferred across VSs, while HIV infection would be transmitted between cells. Chronologically, HIV transfer is an early event occurring immediately after the VS formation, which precedes but does not necessarily lead to transmission, a later event resulting in infection.

1.1 Analysis of the differences between cell-to-cell HIV transfer and transmission by CAp24 intracellular staining

To understand the differences between the terms transfer and transmission, two different coculture models were performed, in which $MOLT_{NL4-3}$, and $MOLT_{BAL}$ cells, were used as effector cells and cocultured, on the one hand, with primary CD4 T cells or, on the other hand, with the $CD4^+/CXCR4^+$ but $CCR5^-$ MT-4 cell line. Primary cells were purified from PBMCs of healthy donors as described in materials and methods, and they present a mostly non-activated phenotype. Cell lines, on the other hand, have acquired the ability to proliferate indefinitely and have an immortalized phenotype that renders them in an actively-dividing state. Analysis of HIV materials associated to these two types of target cells can be measured after intracellular labeling of the CAp24 antigen and analyzed by flow cytometry. Primary cells present a differential morphology than the effector MOLT cell line allowing for a clear distinction by FCS and SSC gating strategies. However, the MT-4 cell line presents a similar morphology to the effector MOLT cells so the gating strategy consisted on the labeling of the target MT-4 cells with the cell tracker DDAO. Once being able to clearly distinguish between the two cell types of the coculture by their correspondent gating strategies, measure of the CAp24 antigen capture was followed. For the evaluation of transfer and transmission events employing the two coculture models, two different time points were set:

1.1.1 Short-time cocultures (2h)

Initially, primary CD4 T cells and DDAO-labeled MT-4 cells were identified following FCS and SSC values and fluorescent staining respectively (Figure 18A). After 2-hours of coculture, CAp24 staining was detected in both the MT-4 cell line and primary CD4 T cells cocultured with $MOLT_{NL4-3}$ and $MOLT_{BAL}$ cells (Figure 18B, left). Addition of the fusion inhibitor C34 did not significantly alter the extent of CAp24 staining to both target cells whereas the blocking anti-CD4 antibody Leu3A abrogated the capture of CAp24 antigen at short incubation times. Transfer of HIV was observed in a coreceptor- and fusion-independent manner as the X4 and the R5 HIV isolates induced similar levels of HIV transfer to target cells regardless of the low or absent CCR5 expression in primary T cells and MT-4 cells respectively.

1.1.2 Long-time cocultures (24h)

A more complex scenario appeared at longer incubation times; MT-4 cells cocultured with $MOLT_{NL4-3}$ cells became totally positive for the intracellular CAp24 antigen with a very high MFI value. In this case, addition of the fusion inhibitor C34 vastly reduced the CAp24 antigen transfer, confirming the existence of a highly efficient process of productive infection acknowledged as HIV transmission in the MT-4 cell line. The remaining fusion-independent CAp24 staining in the presence of C34 was comparable to that observed

after 24 hours of coculture between MT-4 and $MOLT_{BAL}$ cells and may be identified as the coreceptor-independent transfer of HIV materials, which appeared to be partially sensitive to Leu3A.

In contrast, primary CD4 T cells behave in a different manner in long time cocultures with $MOLT_{NL4-3}$ cells. In Figure 18C, primary CD4 T cells cocultured with $MOLT_{NL4-3}$ cells showed an increase in the population of dead cells (defined by low FCS and SSC values; red population in Figure 18C) that was vastly reduced in the presence of Leu3A and C34. Similarly, addition of the fusion peptide C34 completely abrogates syncytia to the same extent than the anti-CD4 mAb Leu3A (Figure 18C, top panels). Even though C34 protected CD4 T cells from dying at the same time it was observed a notable increase in the amount of CAp24 transferred to primary CD4 T cells, suggesting that in the absence of gp41-mediated cell death and fusion, HIV transfer is the only consequence arising from VSs. Consistently, cocultures of primary CD4 T cells with $MOLT_{BAL}$ cells showed a high level of CAp24 transfer to target cells that was unaffected by C34. This fact correlated with the low cell death and fusion observed in all cocultures with $MOLT_{BAL}$ cells due to the low CCR5 expression in primary CD4 T cells. Of note, at 24-hours, the amount of CAp24 accumulated in primary cells by fusion-independent mechanisms was completely sensitive to the anti-CD4 mAb Leu3A, suggesting that primary CD4 T cells show a particular ability to capture HIV particles after engaging in VS.

Taken together, these data suggest that MT-4 cells (and by extension other CD4 cell lines) appear to be a suitable model to study HIV transmission. Conversely, the high level of CAp24 transferred to primary CD4 T cells and their mostly quiescent state are factors that hamper the rapid flow cytometric quantification of infection events making these cells an excellent model to explore the mechanisms involved in early events of HIV transmission, namely HIV transfer.

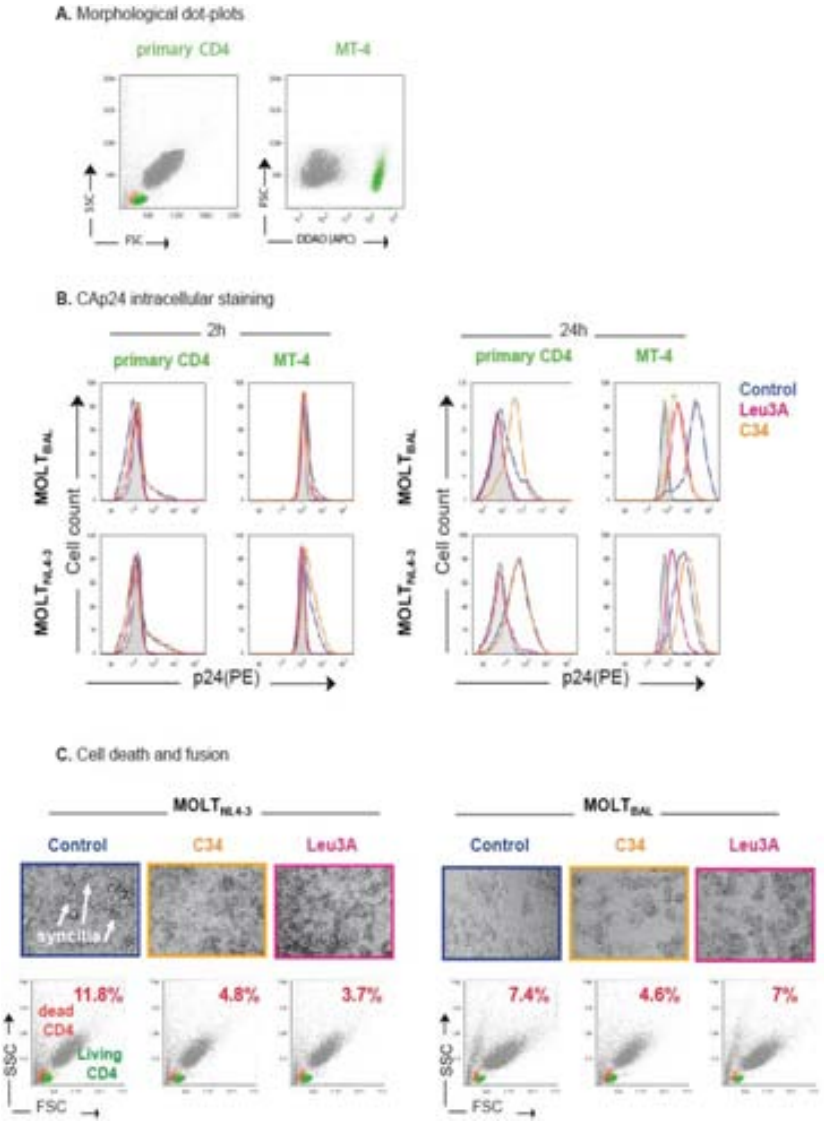

A. Morphological dot-plots
primary CD4
MT-4
SSC
FSC
DDAO (APC)
B. CAp24 intracellular staining
2h
24h
primary CD4
MT-4
Control
Leu3A
C34
Cell count
p24(PE)
C. Cell death and fusion
Control
C34
Leu3A
syncitia
11.8%
dead CD4
Living CD4
4.8%
3.7%
7.4%
4.6%
7%
SSC
FSC

Figure 18

Figure 18. HIV transfer and HIV transmission at the VS. A. $MOLT_{NL4-3}$ and $MOLT_{BAL}$ cells were cocultured either with MT-4 cells or with purified primary CD4 T cells. Target cells were identified by DDAO staining (MT-4) or FCS and SSC values (primary CD4 T cells). **B.** The levels of CAp24 antigen were measured by intracellular staining after 2-hours (left) or 24-hours (right) of coculture. Histograms show CAp24 staining in the green-painted living target cells in the absence (blue) or the presence of the fusion-blocking peptide C34 (orange) or the anti-CD4 antibody Leu3A (dark red). Grey tinted peaks show the staining of uninfected control cocultures. A single representative experiment is shown. **C.** Cocultures of primary CD4 T cells with $MOLT_{NL4-3}$ (left) or $MOLT_{BAL}$ (right) cells were analyzed for the formation of syncytia (top pictures) and the death of primary cells quantified by gating low FCS and SSC events (red) inside the total CD4 T cell population (bottom panels). Effector MOLT cells are shown in grey.

1.1.3 HIV particles are captured by target cells into trypsin-resistant compartments

To test whether HIV particles transferred either at 2 or 24 hours of culture were actually being internalized by the target cells, and not only bound at the cell surface, trypsin was added in the cocultures prior to the CAp24 staining. Trypsinization removed all cell-surface bound HIV particles as it cleaves the CD4 molecules expressed on the cell surface. The best approach to evaluate the internalization of viral particles was using the coreceptor-independent HIV transfer coculture model (i.e. in cocultures of $MOLT_{BAL}$ cells with low or negative CCR5-expressing primary CD4 T or MT-4 cells, respectively). The intracellular expression of CAp24 antigen was

monitored in the presence or absence of trypsin treatment (Figure 19A). Besides, the percentage of HIV particles remaining inside the target cells after trypsin treatment was calculated (Figure 19B). At 2 hours, most of the CAp24 transferred remained insensitive to trypsin treatment whereas at 24 hours about 50% of the antigen transferred was still resistant to trypsin, thus it was concluded that most of the HIV particles were transferred to target cells into trypsin-resistant compartments.

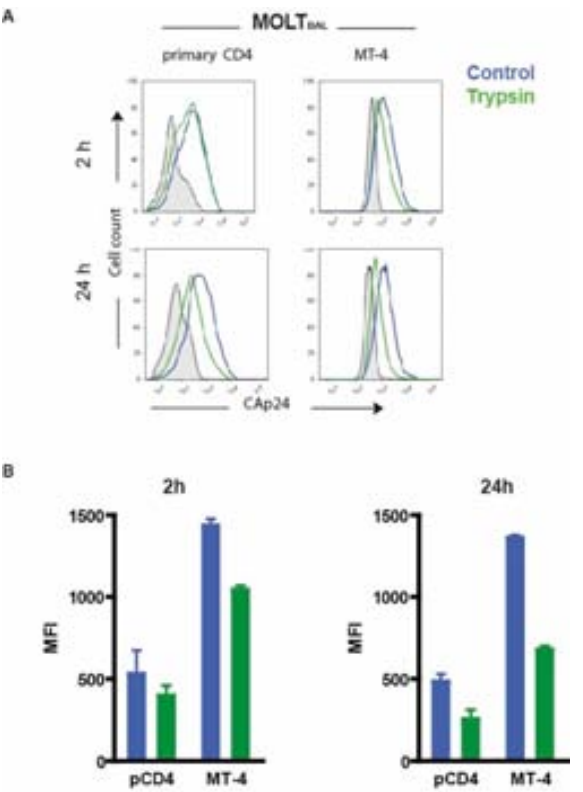


Figure 19

Figure 19. Cell-to-cell HIV transfer into trypsin resistant compartments. A. Intracellular staining of CAp24 antigen was analyzed in cocultures of primary CD4 T cells (left) and MT-4 cells (right) with $MOLT_{BAL}$ cells for 2-hours (upper panels) or 24-hours (lower panels). After incubation, cells were washed with a PBS/EDTA solution and either treated for 10 min with trypsin (green peaks) or left untreated (blue peaks). Grey histograms represent the background staining of target cells cocultured with uninfected MOLT cells. A single representative experiment is shown. **B**. Determination of the percentage of trypsin-resistant CAp24 antigen transferred measured as follows; [(MFI $MOLT_{BAL}$ transfer / MFI $MOLT_{UNINF}$ transfer) / (MFI $MOLT_{BAL}$ transfer trypsin-treated / MFI $MOLT_{UNINF}$ transfer trypsin-treated)]x100. Values are mean ± SD of three different donors.

1.2 Analysis of the differences between cell-to-cell HIV transfer and transmission by membrane transfer

An alternative approach to characterize transport phenomena at the VS is the evaluation of the transfer of membrane lipids from infected MOLT cells to target cells. Membrane transfer to single cells may be the consequence of either hemifusion events between cells, or the transfer of viral particles, carrying cell membrane components dragged during the budding process, that may fuse with or may be endocytosed by target cells. As observed for the CAp24 antigen transfer in Figure 18, membrane transfer was also tightly subjected to Env binding to CD4 as it was completely inhibited with the anti-CD4 blocking antibody Leu3A (Figure 20A). Likewise, transfer of membranes was a fusion-independent process as it was not abrogated in the presence of C34 (Figure 20). When analyzing cell death in cocultures of primary CD4 T cells with $MOLT_{NL4-3}$ cells, the

majority of the dead cells stained positive for the membrane probe (Figure 20, red events), thus indicating that cell death may be a consequence of membrane fusion events which appeared completely blocked in the presence of the fusion inhibitor peptide C34. Altogether, these data showed that, in conditions in which fusion and hemifusion events are completely blocked, membranes were still transferred across the VS. These data corroborate the fusion-independent transfer of HIV particles, this time visualized by membrane transfer analysis. On the other hand, transfer of membranes from $MOLT_{NL4-3}$ or $MOLT_{BAL}$ cells to the MT-4 cell line was lower than the observed for primary CD4 T cells, which reinforced the previous idea that primary CD4 T cells present a unique ability to capture HIV particles (Figure 20A). Of note, only the transfer of membranes from $MOLT_{NL4-3}$ cells to MT-4 cells was partially inhibited by C34, which suggested that low levels of fusion-dependent events are sufficient to induce an efficient infection of cell lines during cell-to-cell HIV transmission.

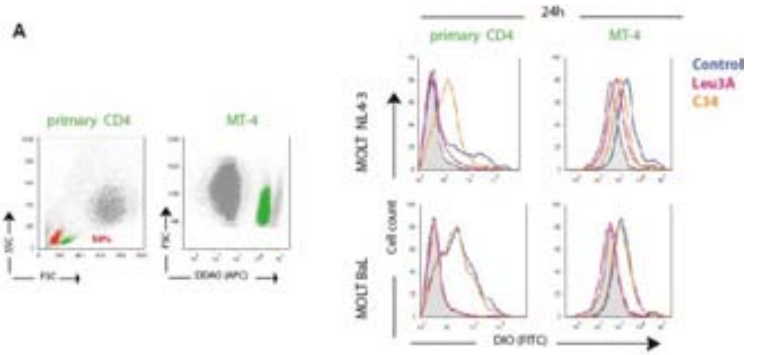


Figure 20

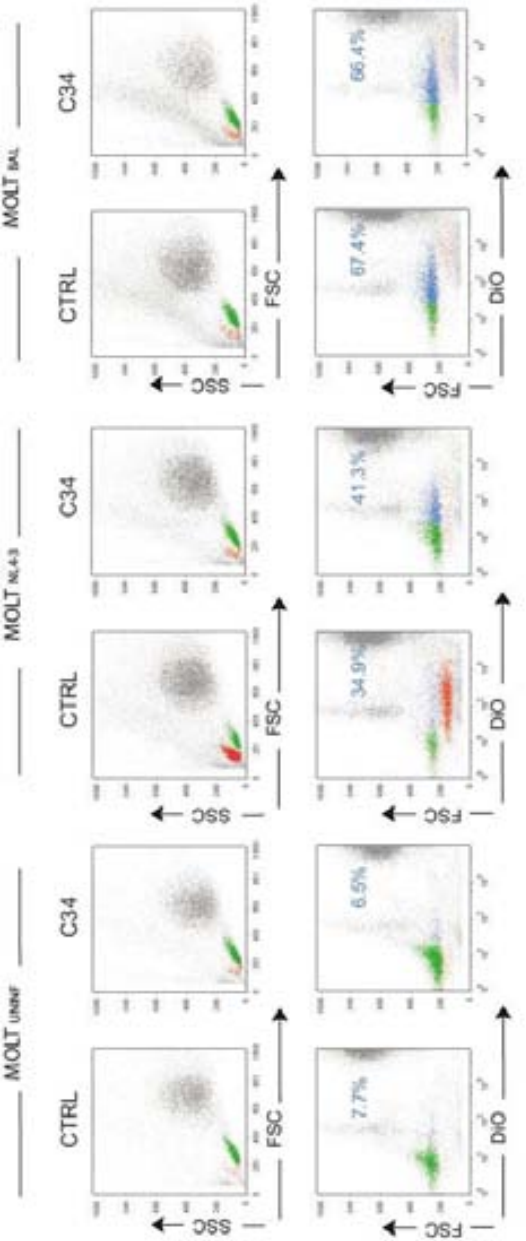


Figure 20. Membrane transfer at the VS and association with cell death in primary cells. Upper dot-plots illustrate the morphology of primary CD4 T cells cocultured with the infected MOLT cells for 24-hours. Living CD4 T cells appear in green and dead CD4 T cells in red. Addition of the fusion inhibitor peptide C34 (orange) was evaluated. Below FCS and DiO staining plots illustrate the transfer of membranes from MOLTNL4-3 cells (grey) to living primary CD4 T cells (blue). The fusion inhibitor peptide C34 was added when indicated. Values correspond to the percentage of DiO positive cells (in blue) in the living CD4 T cell gate.

1.3 HIV transmission into primary CD4 T cells is preferably assessed by a qPCR assay.

Although unstimulated primary CD4 T cells do not produce detectable amounts of HIV particles according to their non-activated state, they support early events of HIV infection that can be measured by quantifying the amount of newly synthesized HIV DNA. Cocultures of $MOLT_{NL4-3}$ and $MOLT_{BAL}$ cells with primary CD4 T cells were stopped at 24 hours and the DNA of the coculture extracted to quantify the total HIV DNA synthesis by qPCR. Amplification of the endogenous CCR5 gene was used to calculate the number of cells present in each sample. The relative proviral DNA was calculated subtracting the background obtained from cocultures at time zero. Concomitantly, HIV DNA quantification was compared with the CAp24 and membrane transfer depicted using the MFI intensities of fluorescence (Figure 21). Comparison showed that CAp24 and membrane transfer processes follow a CD4-dependent but fusion-independent pathway, while proviral DNA synthesis is completely prevented by both CD4 and gp41 blockade, highlighting the differences between HIV transfer and transmission.

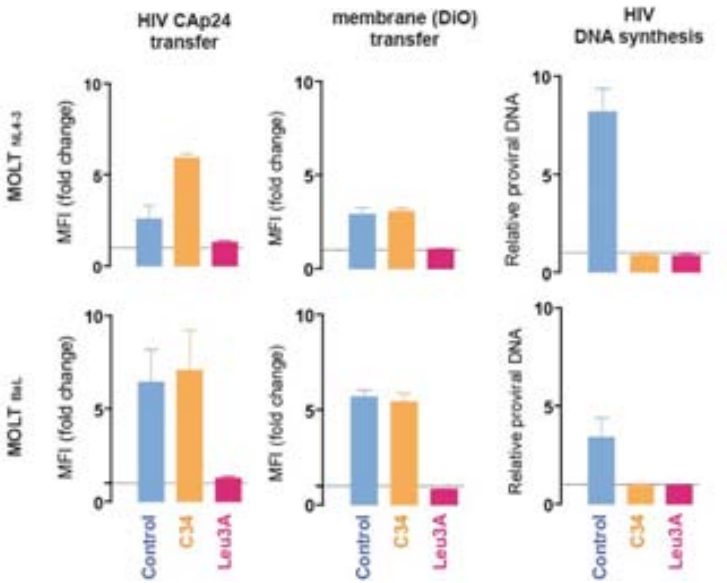


Figure 21. Quantitative analysis of HIV transfer and transmission to primary CD4 T cells. Transfer of CAp24 antigen (left), and membrane (middle) and DNA synthesis (right) after 24-hours of culture between $MOLT_{NL4-3}$ cells (top) or $MOLT_{BAL}$ cells (bottom) and primary CD4 T cells in the absence of inhibitors (blue bars) and the presence of C34 (orange bars) or the anti-CD4 antibody Leu3A (dark red bars). Results show the fold changes from uninfected cocultures in MFI of the living target cells (for CAp24 and DiO transfer) or the fold change from a culture at time zero to measure the relative DNA synthesis into qCD4 T cells. Data are Mean ± SD of three different experiments.

Chapter II: *HIV Transfer and Transmission Between CD4 T Cells Does Not Require LFA-1 Binding to ICAM-1 and is Governed by the Interaction of Env with CD4*

Cell-to-cell HIV transmission requires the formation of cellular conjugates between infected and non-infected cells allowing for a higher infection rate in contrast to the common cell-free viral infection. In this chapter we aimed to evaluate the role of LFA-1 and its ligands ICAM-1 and -3 on cell-to-cell HIV transmission. The functions of these molecules have been previously described in free virus infection of CD4 T cells or in *trans*-infection mediated by DC. However, their role in VSs between infected and uninfected CD4 T cells needed further revision.

The formation of cellular conjugates and subsequent HIV transfer and transmission between productively infected MOLT cells and primary CD4 T cells was not inhibited by a panel of blocking antibodies against ICAM-1, ICAM-3 and LFA-1. Rather, LFA-1 and ICAM-3 mAbs enhanced HIV transfer. Complete abrogation of HIV transfer, transmission and formation of cellular conjugates was only observed when gp120/CD4 interactions were blocked. The dispensable role of LFA-1 in HIV transfer was confirmed using non-lymphoid 293T cells, lacking the expression of adhesion molecules, as HIV producing cells. Moreover, blocking LFA-1 binding to ICAM-1 or ICAM-3 did not inhibit HIV transfer between infected and uninfected primary CD4 T cells. These results suggested that in contrast to other mechanisms of viral spread, HIV transmission between infected and uninfected T cells efficiently occurs in the absence of adhesion molecules. Thus, Env gp120/CD4 interactions are the main driving force behind the formation of cellular conjugates between infected and uninfected CD4 T cells whereas LFA-1 has a secondary role during cell-to-cell HIV transmission.

2.1 Immunophenotype of the effector MOLT cells and the primary CD4 T cell subsets

The notorious role of LFA-1 in HIV attachment [34] and formation of synaptic structures [63] led us to evaluate the role of adhesion molecules in T cell-T cell HIV transmission. To do so, initially the expression of LFA-1 (total and activated forms), ICAM-1 and -3 were confirmed in target purified primary CD4 T cells and effector MOLT cells (Figure 22). All cells stained positive for these antigens with different intensities of expression. Primary CD4 T cells expressed high levels of LFA-1 and low levels of ICAM-3, ICAM-1 and activated LFA-1 where memory CD4 T cells showed higher expression of all antigens as compared to the naïve subset (Figure 22). All MOLT cells (uninfected and NL4-3 or BAL infected) expressed lower levels of LFA-1 (total and activated forms) than primary CD4 T cells, while ICAM-1 and ICAM-3 expression was comparable to that of naïve CD4 T cells (Figure 22). The expression of CD4 and HIV Env was also assessed (Figure 22). Cell surface CD4 staining served to confirm similar levels of expression in naïve and memory primary cells, and the complete downregulation in productively infected MOLT cells (Figure 22). Env expression was comparable in both $MOLT_{NL4-3}$, and $MOLT_{BAL}$ cells.

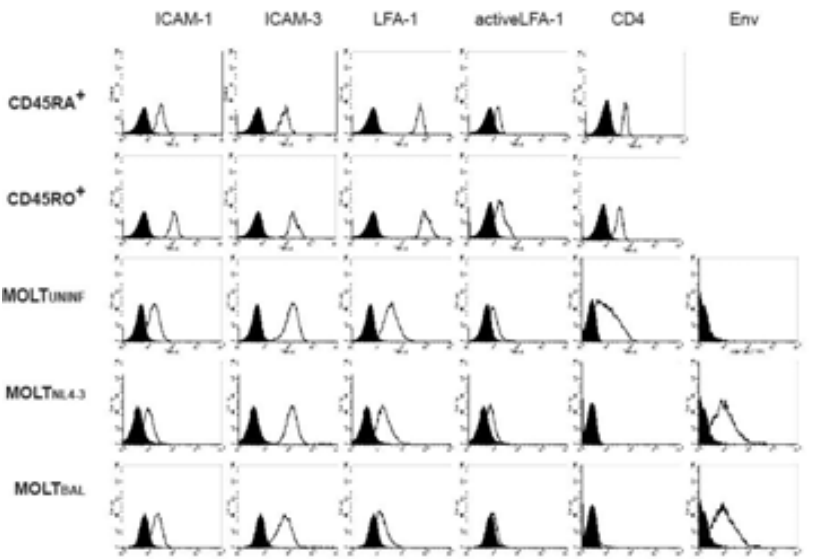
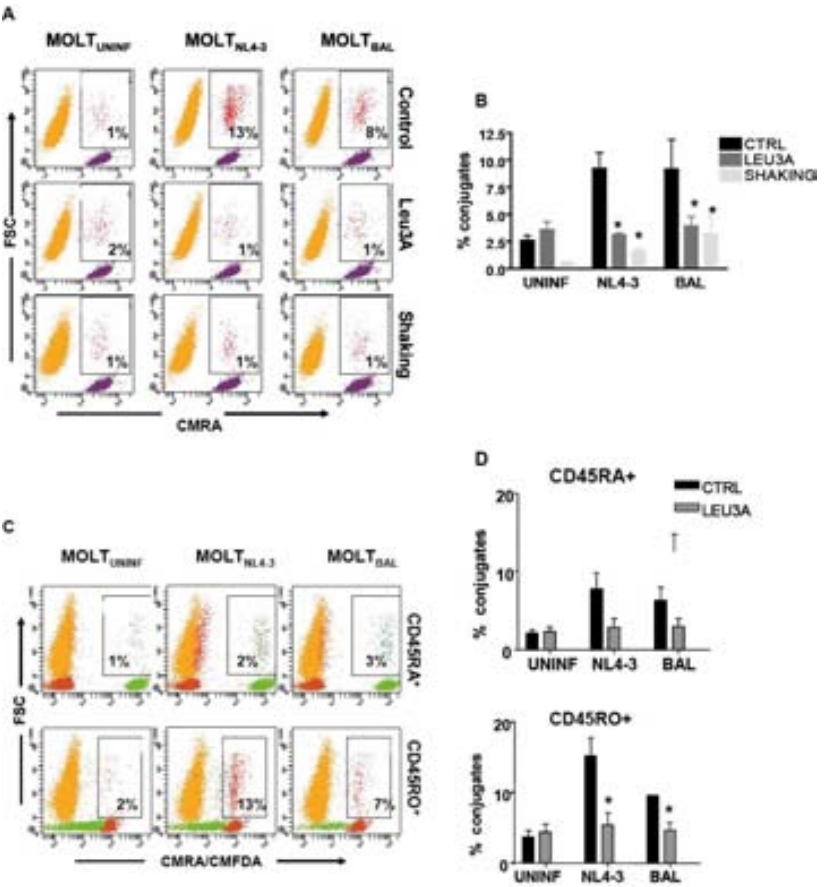


Figure 22. The expression of adhesion molecules on effector and targets cells. Primary CD4 T cells as well as $MOLT_{NL4-3}$ or $MOLT_{BAL}$ cells were analyzed for the expression of ICAM-1, ICAM-3, total LFA-1, the activated form of LFA-1 and CD4. Staining of the cell surface molecules was performed using the monoclonal antibodies RM3A5, 140.11, R7.1, mAb24 and Leu3A respectively. The expression of HIV Env was evaluated in MOLT cells using pooled serums from HIV infected individuals. Histograms show the expression of each individual antigen (empty peaks) with the negative control of staining (solid peaks). Data are representative of a single experiment.

2.2 The role of adhesion molecules on the formation of T cell-T cell conjugates

Since the potential effect of adhesion molecules on HIV transmission was expected to be associated with the initial steps of cellular contacts, a flow cytometry method was developed to quantify cellular conjugates formed between MOLT and fluorescently-labeled CD4T cells. In these experiments, individual cells could be identified by FSC and fluorescence values. Cellular conjugates displayed high levels of fluorescence (given by the labeled CD4 T cells) and FSC values similar to the MOLT cell population (Figure 23A). We employed uninfected MOLT cells as a control of the background levels of Env-independent T cell-T cell contacts. $MOLT_{NL4-3}$ and $MOLT_{BAL}$ cells showed higher percentage of cellular conjugates (13% and 8% respectively) than uninfected cells (1%) (Figure 23A). Regardless of the viral tropism, cellular conjugates were significantly inhibited by the anti-CD4 mAb Leu3A or by continuous shacking during incubation (Figure 23B). In parallel, selectivity of contacts towards a subset of CD4 T cells was analyzed by coculturing memory and naïve CD4 T cells labeled with two different cell trackers at a ratio 1:1 with effector MOLT cells. After 2 hours memory CD4 T cells showed a significant increase in the formation of conjugates either with $MOLT_{NL4-3}$ and $MOLT_{BAL}$ cells as compared to the naïve CD4 T cell subset (Figure 23 C and D).

Conversely, the addition of antagonists of both CXCR4 (AMD3100) or CCR5 (TAK779) coreceptors and the gp41 inhibitor C34 (at concentrations that completely blocked cell-to-cell fusion) failed to inhibit the formation of cellular conjugates (Figure 23E). Similarly, the addition of blocking antibodies against adhesion molecules (LFA-1, ICAM-1 and -3), did not show any significant impact on the formation of cellular conjugates (Figure 23E). As a whole, these data suggested that the expression of HIV Env and CD4 are the main determinants of the formation of cellular conjugates between infected and uninfected CD4 T cells.


A
MOLT UNINF
MOLT NL4-3
MOLT BAL
Control
Leu3A
Shaking
FSC
CMRA
1%
13%
8%
2%
1%
1%
1%
1%
1%
B
% conjugates
CTRL
LEU3A
SHAKING
UNINF
NL4-3
BAL
C
CD45RA+
CD45RO+
CMRA/CMFDA
1%
2%
3%
2%
13%
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CD45RA+
CD45RO+
% conjugates
CTRL
LEU3A
UNINF
NL4-3
BAL

Figure 23

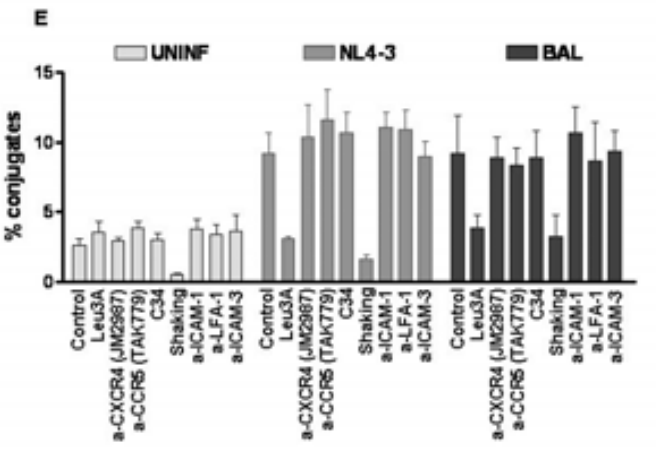

Figure 23. Formation of T cell-T cell conjugates. A. A representative experiment of quantification of cellular contacts between MOLT cells and purified CD4 T cells. Cellular conjugates were defined as events displaying bright fluorescence and FSC values consistent with MOLT cells (gate MOLT-CD4 in the figure). The percentage of CD4 T cells forming conjugates is shown in each plot. **B.** Quantification of cellular conjugates in the absence or the presence of Leu3A, or under continuous shacking condition. **C.** A representative experiment using purified CD45RO and CD45RA cells stained with different cell trackers. Memory and naïve cells were mixed (ratio 1:1) and cocultured with MOLT cells at a final ratio of coculture of 1:1. The percentage of naïve (top) and memory (bottom) CD4 T cells forming conjugates is shown. **D.** Quantification of cellular conjugates formed by CD45RA (top) and CD45RO (bottom) cells, in the absence or the presence of Leu3A. Asterisks in panel E denote significant differences between memory and naïve cells. **E**. Quantification of cellular conjugates in the presence of the following inhibitors: mAbs against the adhesion molecules ICAM-1 (RM3A5), ICAM-3 (140.11) and LFA-1 (R7.1), coreceptor antagonists AMD3100 and TAK779 (all at 10 μg/ml), gp41 inhibitor C34 (1 μg/ml) or Leu3A (5μg/ml). Data are Mean±SD of 3 independent experiments including 3 different donors.

2.3 The role of adhesion molecules on cell-to-cell HIV transfer and transmission between CD4 T cells.

Recently, Jolly et al [82] have suggested that during HIV spread between CD4 T cells, adhesion molecules may modulate not only the formation of cellular conjugates but also the consequences of VS [82]. To address this possibility in the MOLT-primary CD4 T cell coculture system, we tested the effect of antibodies blocking adhesion molecules in an assay that measures transfer of HIV particles from infected to uninfected T cells. The blockade of the alpha or beta chains of LFA-1 (CD11a and CD18, respectively), the activated form of LFA-1, and ICAM-1 or ICAM-3 did not show any inhibitory effect on the transfer of HIV particles, measured by intracellular staining of the CAp24 antigen into target cells (Figure 24A). In the search of other adhesion components expressed on the cell surface, which could be involved in the formation of cellular conjugates or HIV attachment, we have also evaluated the role of CD147 and CD29. The CD147, a type I integral membrane glycoprotein has been identified as a cell surface receptor for extracellular cyclophilin A [173] which is known to be incorporated into newly synthesized virions during the budding process [174]. The CD29 integrin has been associated to cell aggregation mechanisms in lymphocytes and hence its role in cell-to-cell transmission was studied herein [175]. Nonetheless, blocking antibodies against these latter molecules did not inhibit HIV transfer; rather a significant increase was observed in the presence

of the anti-CD147 antibody (Figure 24A). All together, HIV transfer was not affected in the presence of blocking antibodies against a panel of adhesion molecules.

The unessential role of the tested adhesion molecules in cell-to-cell HIV transfer lead us to evaluate further steps of the HIV transmission route. As described in chapter I, detection of HIV provirus by RT-PCR into primary CD4 T cells cocultured with infected MOLT cells was a reliable measure of infection of primary CD4 T cells. Hence, the evaluation of the presence of adhesion molecules in HIV transmission needed to be further characterized. Cocultures of $MOLT_{NL4-3}$ cells with primary CD4 T cells were halted at 24 hours to extract DNA and perform a real time qPCR analysis to quantify the number of HIV copies/cell. Again, addition of blocking antibodies against the LFA-1, ICAM-1 and ICAM-3 as well as the Fab fragment of the latter and the ICAM-1 soluble molecule did not show inhibition of the HIV infection as compared to the control coculture (Figure 24B). The background of the HIV provirus that comes from the MOLT cells was subtracted from all samples using a coculture stopped at time zero.

All together, our data suggested that mAbs against adhesion molecules do not block HIV transfer and transmission due to their inability to inhibit the formation of cellular conjugates.

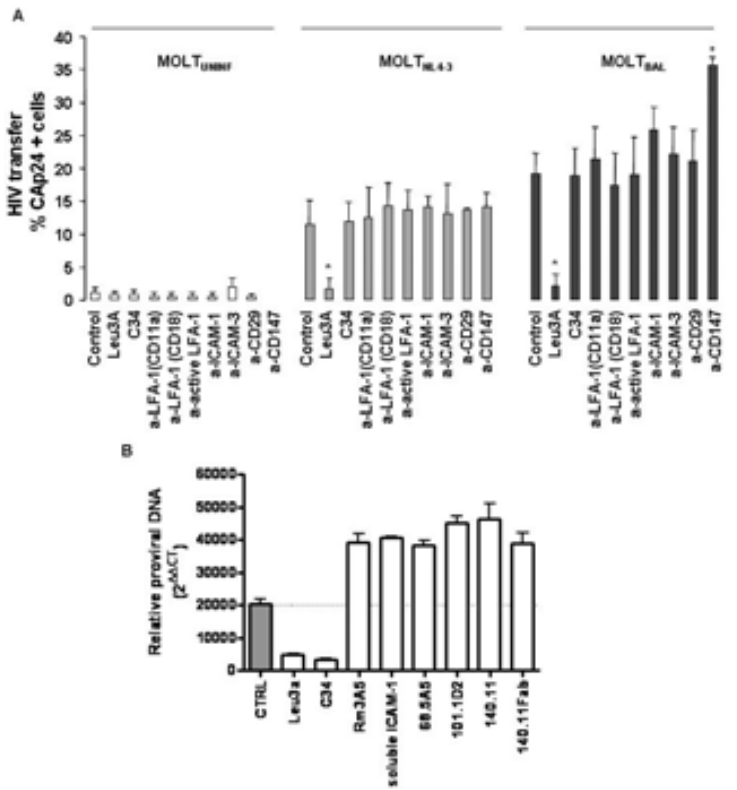

Figure 24. The role of adhesion molecules on T cell-to-T cell HIV transfer & transmission. A. Transfer of HIV particles after 2 h of coculture of primary CD4 T cells with uninfected MOLT (white bars), MOLT$_{NL4-3}$ (grey bars) or MOLT$_{BAL}$ cells (dark bars) was assessed in the absence (Control) or the presence of blocking antibodies against the indicated cell surface molecules. **B.** Cocultures of primary CD4 T cells with MOLT$_{NL4-3}$ cells were incubated for 24 hours prior to DNA extraction. HIV DNA synthesis was measured using real time qPCR relative to cocultures stopped at time zero. Concentrations were 10 μg/mL for all mAbs against cell surface molecules, except for Leu3A (5 μg/mL) and C34 (1 μg/mL). Data are Mean± SD of 3 independent experiments employing cells of 3 different donors. Asterisks indicate a significant difference in HIV transfer/transmission as compared to control cocultures.

2.4 Analysis of HIV transfer in an LFA-1, ICAM-1 and ICAM-3 deficient system

The failure of antibodies against adhesion molecules to inhibit the formation of cellular conjugates and the blocking effect of Leu3A suggested that Env gp120 binding to CD4 is the main driving force behind the formation of T cell-to-T cell VSs. From these observations, we suggested that T cell-to-T cell HIV transmission might take place in the absence of LFA-1 interactions with ICAMs. To further investigate the requirements of LFA-1 function in HIV transfer, we used 293T cells as effector cells. First, we confirmed that these cells do not show detectable expression of LFA-1, ICAM-1, -2 and -3 on their surface as measured by flow cytometry (Figure 25A). Second, 293T cells were transfected with plasmids coding for an Env deficient genome of HIV (Δenv) together with an Env/Rev cassette (NL4-3 isolate) as illustrated in materials and methods. Of note, 293T cells transfected with the plasmid ΔEnv alone were used as a control of potential Env-independent nonspecific transmission. Transfected 293T cells cocultured with primary CD4 T cells showed specific HIV transfer from Env-expressing 293T to CD4 T cells by a mechanism similar to that observed for MOLT infected cells: it was abrogated by Leu3A and unaffected by the addition of C34 (Figure 25B), reasserting that the binding of LFA-1 to its ligands is not necessary to observe an efficient HIV transfer.

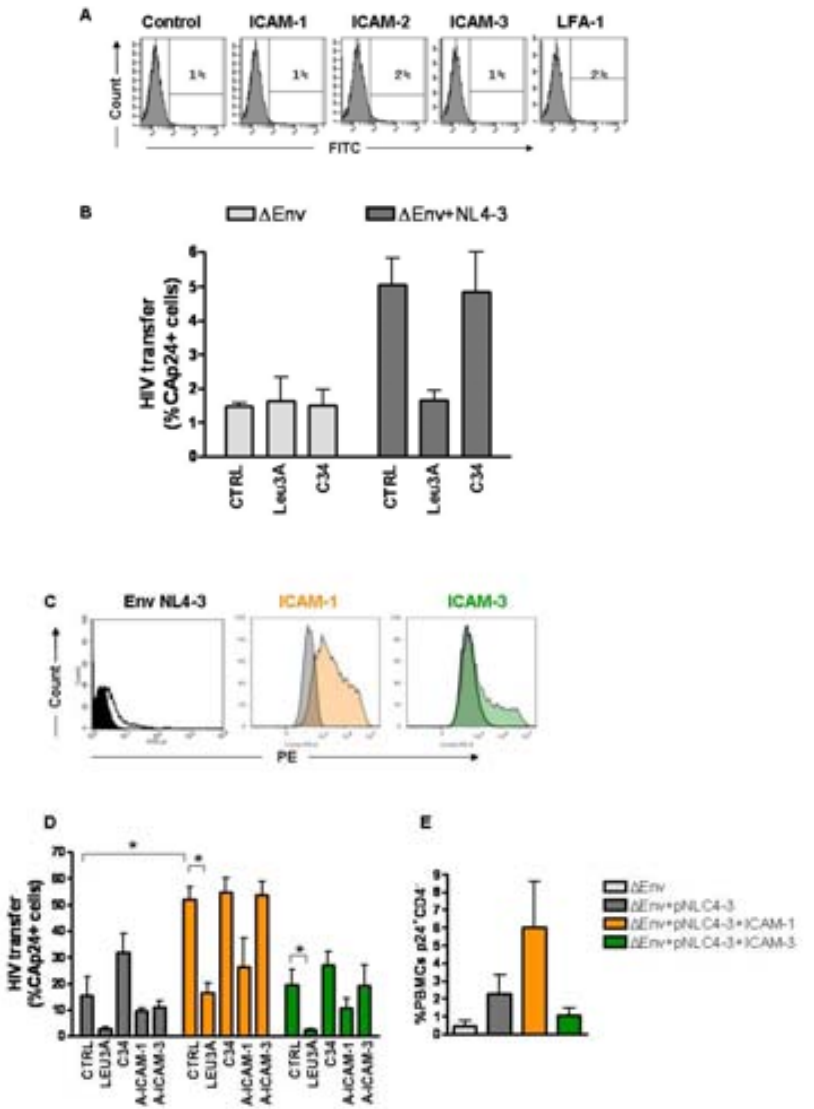
A
Control
ICAM-1
ICAM-2
ICAM-3
LFA-1
Count
1%
1%
2%
1%
2%
FITC
B
ΔEnv
ΔEnv+NL4-3
HIV transfer
(%CAp24+ cells)
CTRL
Leu3A
C34
CTRL
Leu3A
C34
C
Env NL4-3
ICAM-1
ICAM-3
Count
PE
D
HIV transfer
(%CAp24+ cells)
CTRL
LEU3A
C34
A-ICAM-1
A-ICAM-3
E
%PBMCs p24+CD4+
ΔEnv
ΔEnv+pNLC4-3
ΔEnv+pNLC4-3+ICAM-1
ΔEnv+pNLC4-3+ICAM-3

Figure 25

Figure 25. LFA-1 independent cell-to-cell HIV transfer. Panel A depicts the expression of the indicated adhesion molecules on the surface of the 293T cell line. The percentage of positive cells is indicated in each histogram. Control histogram corresponds to background of staining. **Panel B** shows HIV transfer from 293T cells, transfected to produce HIV particles, to target CD4 T cells in the absence (light bars) or presence (grey bars) of NL4-3 Env expression. The effect on the addition of blocking antibodies against CD4 (Leu3A) and the gp41 inhibitory peptide C34 was also tested. Data (Mean± SD) were obtained using cells from 3 different donors. Asterisk indicates a significant inhibition in HIV transfer in the presence of Leu3A within NL4-3 Env transfected 293T cells. **Panel C** illustrates the expression of Env, ICAM-1 and ICAM-3 on the surface of cells transfected to produce HIV particles in the absence or the presence of a vector coding for ICAM-1 or ICAM-3, respectively. **Panel D** shows HIV transfer from 293T cells in the absence (grey lines) or the presence of a plasmid coding for ICAM-1 (orange lines) or ICAM-3 (green lines) to primary $CD4^+$ T cells. **Panel E.** PBMCs from healthy donors were infected with equal amounts of HIV particles obtained from supernatants of 293T cells transfected either with an HIV plasmid alone or in combination with ICAM-1 or ICAM-3 plasmids. Cell-free infections were performed in PHA-activated PBMCs for 3 days. At 3 days post-infection, staining of CD4 and intracellular CAp24 of infected PBMCs was conducted. Productively infected PBMCs were considered those events with low CD4 and high CAp24 staining. Data are Mean±SD of 3 different donors. Asterisk indicates a significant inhibition in HIV transfer.

Since 293T cells have been used as a standard model to evaluate the role of ICAM-1 in HIV infection [34, 89], we added to the cotransfection either a plasmid coding for ICAM-1 or ICAM-3. The expression of HIV Env, ICAM-1 and ICAM-3 was assessed into 293T cells by flow cytometry after 24 hours of transfection (Figure 25C). Transfer of HIV particles in the presence of ICAM-1 showed a significant increase ($p<0.05$) compared to the control or the ICAM-3 transfected cells (Figure 25D). Besides, addition of the blocking antibody against ICAM-1 (Rm3A5) reduced the HIV transfer to the levels achieved in the control cocultures. HIV transfer mediated by ICAM-1 expressing 293T cells was not modified by C34, and was inhibited by Leu3A (Figure 25D). Thus, ICAM-1 may be modulating the HIV transfer although is not an indispensable component for this process. The different role of ICAM-1 observed in HIV transfer from MOLT or from 293T cells to CD4 T cells could be due to a different balance between the expression of ICAM-1 and HIV Env on the surface of the transfected 293T cells (26 ±6 % ICAM-1 expressing cells vs. 14±3% Env expressing cells, with relative fluorescence intensity (RFI) values of 4.3±2 and 2.1, respectively). In fact, 293T cells expressed lower levels of Env than ICAM-1 both in terms of percentage of positive cells and RFI. In contrast, the expression of ICAM-1 is low in MOLT cells, which in turn express higher levels of HIV Env (RFI values of 2.7 and 7 for ICAM-1 and Env, respectively in $MOLT_{NL4-3}$ infected cells, Figure 22).

Infection of PBMCs with viruses produced in 293T cells in the presence or absence of ICAM-1 or ICAM-3 expression was assessed. Results depicted in Figure 25E showed an increase in the infection of PBMCS with ICAM-1 bearing viruses as compared to the control or ICAM-3. These comparative results were used as a control of the different infectivity of viruses in a cell-free environment as described in [34, 89].

In summary, these results reassert the previous observation that LFA-1-ICAM-1 interaction are not necessary to observe cell-to-cell HIV transfer nor transmission as assessed by blocking antibodies or in a system that lack the expression of these adhesion molecules.

2.5 Selectivity of LFA-1 independent HIV transfer

Since the expression of several adhesion molecules in memory CD4 T cells was higher compared to the naïve subset (Figure 22), the active contribution of LFA-1 to the process of HIV spread has been associated to the higher susceptibility of CD4 CD45RO T cells to HIV attachment and infection [176]. Therefore, we sough to evaluate whether LFA-1-independent HIV transfer between CD4 T cells might affect the target cell selectivity. In this set of experiments, after coculture with HIV producing cells, we gated memory ($CD45RO^+$) and naïve ($CD45RO^-$) cells and then we calculated the percentage of CAp24 positive cells in each subset. Figure 26A (left) showed that memory CD4 T cells had significantly higher levels of

CAp24 than naïve cells when cocultured with both $MOLT_{NL4-3}$ and $MOLT_{BAL}$ cells ($p<0.05$). This selectivity of HIV transfer towards the memory subset was independent of LFA-1 binding to ICAM-1, as it was not modified by the addition of the anti-ICAM-1 mAb RM3A5 (Figure 26A, right).

On the other hand, we used the HIV producing 293T cells transfected or not with the plasmid coding for ICAM-1 to study the LFA-1-independent selectivity of HIV transfer. The selective transfer to memory cells was still observed in the absence of adhesion molecules (Figure 26B, light grey bars). Once more, memory T cells (RO^+) showed significant higher values of CAp24 content than naïve cells (RO^-) (Figure 26B, left). High levels of ICAM-1 expression in effector cells significantly increased the efficiency of HIV transfer to both naïve (1.2 fold) and memory T cells (2.1 fold) (Figure 26B, left). Consistent with a specific role of ICAM-1 in this cellular model, the anti-ICAM-1 mAb Rm3A5 reverted the net increase of HIV transfer and the higher selectivity towards memory cells (RO^+) induced by ICAM-1 expression although this antibody was unable to completely block the total HIV transfer (Figure 26B, right). These data supported the idea that the role of ICAM-1 is cell type dependent and provided a positive control for the inhibitory effect of the anti-ICAM-1 mAb Rm3A5.

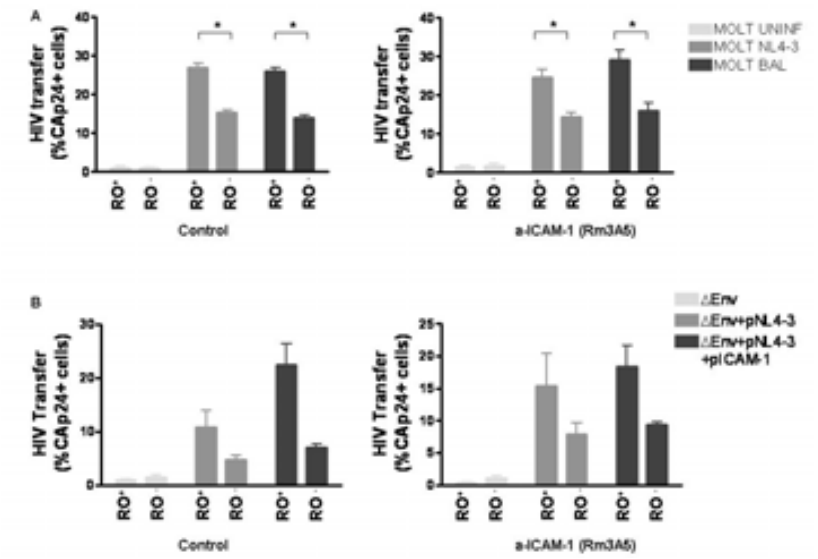

Figure 26. Preferential LFA-1-independent transfer of HIV particles to RO⁺ cells during T cell-T cell synapses. A. $MOLT_{NL4-3}$ (grey bars) and $MOLT_{BAL}$ (dark bars) cells or uninfected MOLT cells (light bars) were cultured with purified primary CD4 T cells. After 2 h of coculture, cells were stained with anti-CD45RO and anti-HIV CAp24 antibodies. Analysis of HIV transfer in the absence (left graph) or the presence (right graph) of the Rm3A5 blocking mAb against ICAM-1 was performed after gating separately CD45RO and CD45RA CD4 T cells. **B.** HIV transfer from 293T cells transfected with an Env defective (Δenv) and an NL4-3 Env plasmids, to naïve (RO^-) and memory (RO^+) target cells in the absence (light bars) or presence (grey bars) of ICAM-1 expression. HIV transfer was again measured in the absence (left graph) or the presence (right graph) of the RM3A5 blocking antibody against ICAM-1. Values are Mean± SD of 3 experiments performed with cells from 3 different donors. Asterisks denote significant differences in HIV transfer to the different T cell subsets (Panel A and B). Significant differences intra-subsets induced by ICAM-1 expression are also indicated by asterisks in panel B, while ns denote no statistical significance.

2.6 HIV transfer between primary CD4 T cells

To clearly define the role of ICAM-1 in HIV transfer between primary CD4 T cells, we purified productively infected CD4 T cells from infected cultures of PBMC and used them as effector cells. First, we determined the expression of LFA-1, ICAM-1 and ICAM-3 in gated $CD3^+CD8^-CD4^-$ T cells of infected PBMC cultures, which represent the productively infected cell population [82]. The expression of the adhesion molecules was compared to the $CD3^+CD8^-CD4^+$ cells of the same cultures, finding no significant differences (Figure 27A). Next, purified productively infected $CD3^+CD8^-CD4^-$ T cells were cocultured with unstimulated uninfected primary CD4 T cells. Transfer of HIV was again detected in both naïve and memory subsets, being significantly ($p<0.05$) higher in the latter and significantly ($p<0.05$) inhibited by Leu3A but not C34 (Figure 27B). Similarly to MOLT cells, the addition of the blocking ICAM-1, LFA-1 or ICAM-3 mAbs did not inhibit HIV transfer; rather significant increases were induced by the 140.11 anti-ICAM-3 or the R7.1 anti-LFA-1 antibodies (Figure 27B). To address the role of antibody mediated cross linking and signaling of these enhancing effects, we compared the effect of Fab fragments of the ICAM-3 mAb 140.11 or a monomeric soluble form of ICAM-1 with the effect of whole IgGs. The increase in HIV transfer induced by the anti-LFA-1 R7.1 IgG was significantly lost when LFA-1 was blocked using soluble ICAM-1 (Figure 27B). Similarly, significant differences were observed between the enhancing effect of the anti-ICAM-3

140.11 IgG and its Fab fragment, although the latter still retained some ability to increase HIV transfer (Figure 27B). None of the inhibitors used but the anti-CD4 mAb Leu3A, inhibited HIV transfer. In summary, these data supported the idea that binding of the HIV Env gp120 to CD4 governs HIV transfer between CD4 T cells in the absence of LFA-1 interactions with ICAM-1 or -3. Nevertheless, signaling through these adhesion molecules may modulate both the extent and the target cell selectivity of viral transfer.

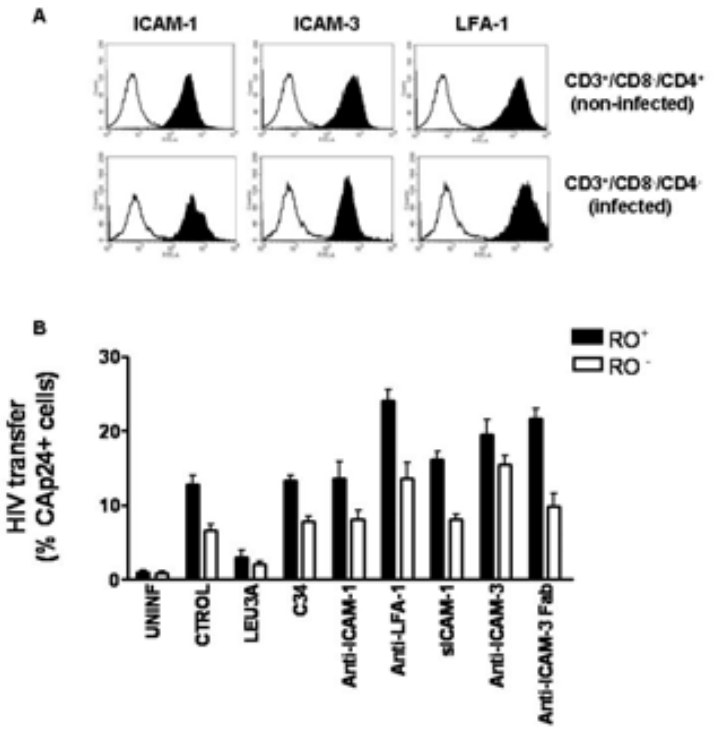

Figure 27

Figure 27. The role of adhesion molecules in HIV transmission between primary CD4 T cells. A. The expression of the indicated adhesion molecules was analyzed in the surface of T cells from a representative NL4-3 infected PBMC culture. Cells were gated as $CD3^+CD8^-CD4^+$ PBMCs (non-productively infected cells, upper histograms) or $CD3^+CD8^-CD4^-$ PBMCs (productively infected cells, lower histograms). Histograms show a representative experiment displaying the expression of each individual antigen (solid peaks) with the negative control of staining (empty peaks). **B.** Purified productively HIV infected $CD3^+CD8^-CD4^-$ PBMCs were cocultured with CMFDA-labeled primary unstimulated CD4 T cells. After 24 hours of coculture, cells were stained with anti-CD45RO and anti-HIV CAp24 antibodies. HIV transmission was measured in both memory (RO^+, solid bars) and naïve (RO^-, empty bars) subsets in the presence of Leu3A, C34 and a panel of blocking agents against adhesion molecules; whole IgGs against ICAM-1, LFA-1 and ICAM-3, soluble ICAM-1 or the Fab fragments of the anti-ICAM-3 mAb 140.11 at 10 μg/mL. Values are Mean± SD of data corresponding to up to 6 different donors.

Chapter III: *The contribution of Trogocytosis in Cell-to-Cell HIV Transmission Between CD4 T Cells*

Several reports have documented that lymphocytes can extract surface molecules through the 'immunological synapse' from the antigen-presenting cells to which they are conjugated, a phenomenon known as trogocytosis. According to these, we have explored the role of trogocytosis as a possible mechanism exploited for HIV to travel across VSs.

First, in cocultures of chronically infected MOLT cells with 293T cells expressing the fusion protein CD4-GFP, transfer of the CD4-GFP receptor and membrane patches were observed from uninfected to infected T cells. Second, 293T cells transfected either with an HIV Env alone (NL4-3 or the fusion defective mutant 41.2) or in combination with a ΔEnv plasmid were cocultured with primary CD4 T cells and transfer of membrane components was explored in the HIV transmission direction. Membrane transfer from infected to uninfected T cells occurred in a fusion-dependent manner as it was blocked in the presence of the fusion inhibitor C34 or the fusion-defective 41.2 Env.

These results concluded that trogocytosis although associated with VSs it does not explain the transfer of HIV between cells. Instead, viral particles need to be extracellularly released and then captured/internalized by target cells. As trogocytosis was only operating on the opposite direction of the HIV transmission, the consequences that this mechanism might have during cell-to-cell HIV spread need to be further investigated.

3.1 CD4 molecules participate in trogocytic events after the formation of antibody-induced ISs.

Trogocytosis could be triggered by adding the appropriate antibodies in a coculture between T cells and FcR-expressing cells [172]. The murine P815 cell line naturally expresses the FcR receptors, which were stably transfected with a vector expressing the human CD4 molecule fused to GFP. Monoclonal antibodies triggering trogocytosis in human T cells have been previously described [119]. Among them, the anti-CD3 and anti-CD28 mAbs were chosen for their properties of triggering trogocytosis upon FcR crosslink with a high efficiency. Therefore, a cocktail of anti-CD3 and anti-CD28 antibodies was used to coat primary isolated CD8 T cells in order to induce trogocytosis upon contact with the FcR expressing P815 cells. Coculture of the CD3/CD28 coated CD8 T cells with FcR-expressing P815 cells, induced trogocytosis of CD4-GFP molecules by a mechanism previously described as "*re-directed trogocytosis*" [177]. Flow cytometry analysis illustrated the transfer of the CD4-GFP molecule from P815 to primary CD8 T cells. The levels of CD4-GFP expression in the target CD8 population did not reach the same fluorescence intensity observed in the effector P815 cell line since not all cell-surface CD4 molecules are transferred by trogocytosis but only the ones that cluster at the synapse (Figure 28). Capture of CD4-GFP was inhibited when P815 cells were pre-incubated with the blocking anti-FcR antibody 2.4G2 or using non-coated CD8 T cells as target cells (Figure 28).

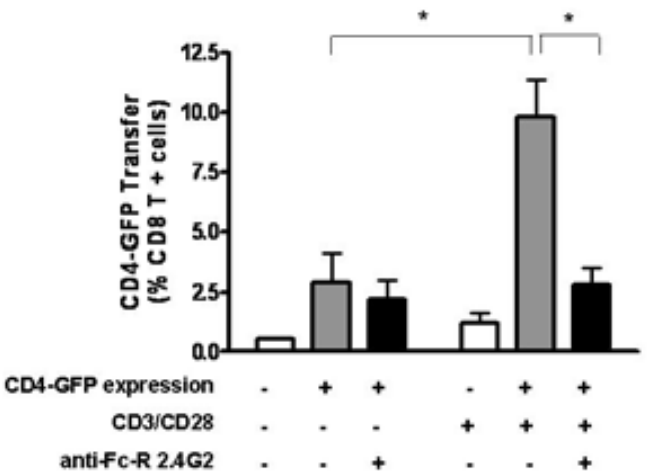

Figure 28. Primary CD8 T cells capture CD4 from P815 CD4-GFP cells during redirected trogocytosis. CD8 T cells isolated from human PBMC were coated or not with anti-CD3+anti-CD28 mAbs prior to incubation with P815 cells transfected or not with human CD4 fused to GFP. Capture of CD4-GFP was analyzed by flow cytometry by determining the population of GFP positive cells on gated CD8 T cells. P815 CD4-GFP cells were cocultured with primary CD8 T cells in the absence (red bars) or the presence (pink bars) of the anti-FcR receptor 2.4G2. CD8 T cells cocultured with P815 not expressing the CD4GFP construct were used as negative controls (grey bars). Asterisks denote significant inhibitions ($p<0.05$).

3.2 Trogocytosis as a possible mechanism for cell-to-cell HIV transmission

We have observed CD4 transfer to CD8 T cells in an antibody-induced model for CTL synapse, suggesting that T cells may support trogocytic events wherein CD4 molecules can be transferred form one cell to another. Therefore the next step was to evaluate the role of trogocytosis in the context of a VS formed between infected and uninfected CD4 T cells in order to explore the involvement of this mechanism on cell-to-cell HIV transfer.

3.2.1 Transfer of membranes operates from uninfected to infected CD4 T cells

Initially, the transfer of membrane fragments from uninfected to infected cells was determined using 293T cells expressing the CD4-GFP construct. These cells were cocultured with DDAO-labeled MOLT cells. The transfer of CD4-GFP was readily observed from 293T cells to $MOLT_{NL4-3}$ and $MOLT_{BAL}$ cells after 2 hours of incubation (Figure 29A) as measured by flow cytometry analysis. Infected MOLT cells became positive for CD4 but with a low intensity of expression as compared to the 293T cells stably transfected with the CD4-GFP construct. Trogocytic capture of CD4 was blocked in the presence of the anti-CD4 mAb Leu3A or the antig-Envgp120 IgGb12, but was not inhibited with the fusion inhibitor peptide C34 (Figure 29A), suggesting that only Env-CD4

mediated contacts are sufficient for trogocytic events to occur at the infected T - uninfected T cell VS. Uninfected MOLT cells were used as a control of Env-independent trogocytic transfer.

Since trogocytosis does not only imply transfer of single cell-surface receptors but whole membrane fragments, trogocytosis of entire membrane patches was assessed. For this purpose, 293T CD4-GFP cells were uniformly stained with the membrane probe DiI and the transfer of membrane patches was measured into DDAO-labeled MOLT cells. Transfer of DiI-labeled membranes was observed into $MOLT_{NL4-3}$ and $MOLT_{BAL}$ cells but not in the uninfected MOLT cells used as negative control (Figure 29B). Moreover, DiI transfer was inhibited in the presence of Leu3A (Figure 29B). Cell membrane functions as a barrier that physically separates the intracellular components from the extracellular environment. To establish whether membrane transfer was a result of cell-cell fusion, the exchange of cytoplasmatic components between the VS counterparts was also assessed. For the experimental design, infected and uninfected MOLT cells were cocultured with 293T CD4-GFP cells previously stained with the intracellular cell tracker CMRA (Figure 29C). These dyes do not leak out of the cells over time, so the detection of cytoplasm dye transfer in conjugates would reflect the exchange via the fusion pore formed between cells. Infected MOLT cells did not capture cytoplasm dye during Env-mediated contacts (Figure 29C) confirming that trogocytosis only involves the exchange of cell-surface components as a result of cellular contacts

in a process that does not require cell-cell fusion. All together, these results suggested the existence of trogocytic mechanisms at the VS from the uninfected towards the infected HIV transmission direction.

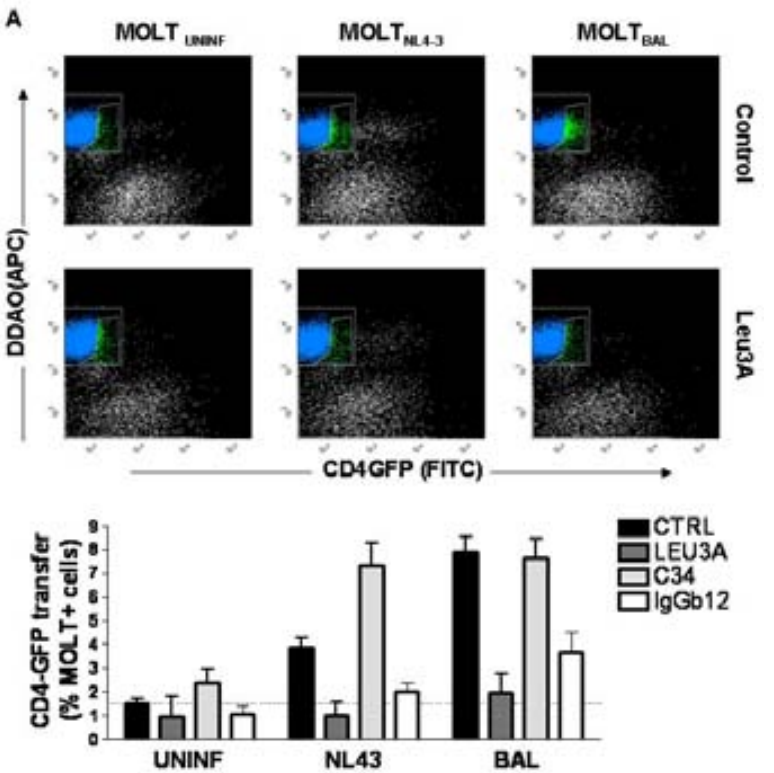

Figure 29

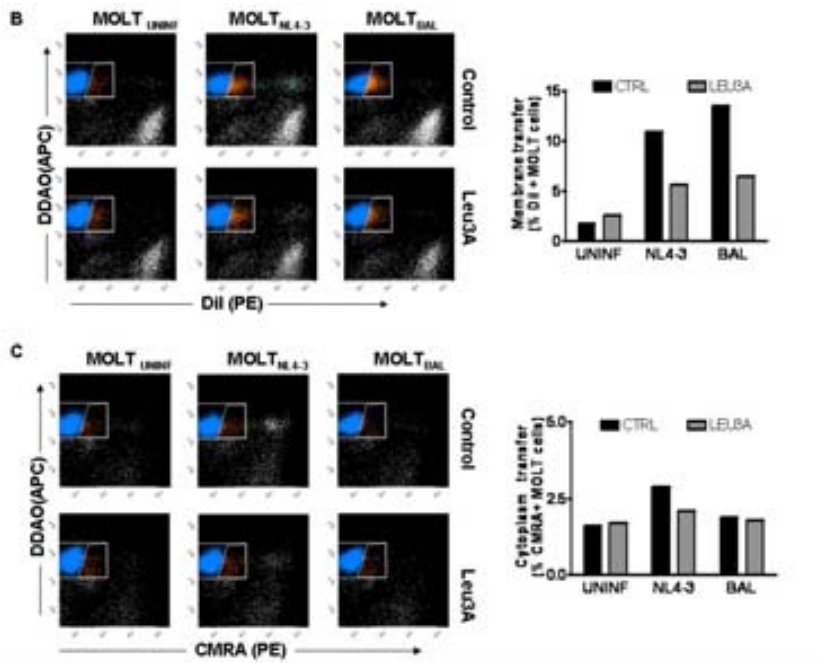

Figure 29. Trogocytosis operates from the uninfected towards infected CD4 T cell direction. Panel A. The trogocytic transfer of membranes from uninfected to infected cells was determined in cocultures of 293T cells expressing a CD4-GFP with $MOLT_{NL4-3}$ or $MOLT_{BAL}$ cells in the absence (Control) or the presence of the indicated inhibitors Leu3A, C34 and the anti-Env IgGb12 nAb at concentrations that completely inhibit VS formation. **Panel B.** 293T CD4-GFP expressing cells were labeled with the DiI membrane probe and cocultured with $MOLT_{NL4-3}$ or $MOLT_{BAL}$ cells in the absence (black bars) or the presence (grey bars) of the anti-CD4 antibody Leu3A. **Panel C.** 293T CD4-GFP expressing cells were intracellularly labeled with the cell tracker CMRA and cocultured with $MOLT_{NL4-3}$ or $MOLT_{BAL}$ cells in the absence (black bars) or the presence (grey bars) of the anti-CD4 antibody Leu3A. Uninfected MOLT cells were used as negative controls.

3.2.2 Transfer of membrane components from infected to uninfected CD4 T cells.

Then, trogocytosis operating at the HIV transmission direction was further assessed as a possible mechanism that would partially explain HIV transfer across the VS. In chapter I, transfer of membranes from infected MOLT cells to uninfected CD4 T cells was observed in a fusion-independent manner. In this chapter, membrane transfer from infected to uninfected T cells was determined using a different experimental setting. The exchange of membrane fragments from infected to uninfected cells was studied using Env-expressing 293T cells labeled with the DiI membrane probe previous to the coculture with primary CD4 T cells. To avoid the fusion dependent membrane exchange, we used an Env mutant (41.2) devoid of fusogenic activity [178]. This Env mutant was able to bind to CD4 and promote HIV transfer, to the same extent than a wild-type NL4-3 Env, when cotransfected with an ΔEnv HIV backbone (Figure 30A). Addition of the fusion inhibitor peptide C34 did not modify the extent of membrane transfer in any case. Thus, HIV transfer measured by tracking membrane transfer to uninfected CD4 T cells followed a fusion-independent pathway as previously observed in chapter I using a different coculture model (Figure 20).

From the previous results where Env gp120-CD4 interaction determined the transfer of membranes across the VS, we hypothesized that only the cell-surface expressed Env binding to the

CD4 receptor expressed on the surface of target cells should be sufficient to allow the transfer of membrane components across a "synapse-like structure" without HIV particle formation. Thus we evaluated the transfer of membranes (trogocytosis) in the HIV transmission direction using an elegant experimental design. Either the wt NL4-3 Env or the 41.2 Env mutant alone were transfected into 293T cells. After 24 hours, transfected cells were stained with the membrane probe DiI prior to the coculture with primary CD4 T cells. Membrane transfer was determined at 24 hours into gated primary CD4 T cells. Results depicted that 41.2 mutant, when transfected alone, promoted background levels of membrane transfer to target cells, which were not significantly modified by the addition of Leu3A or C34 (Figure 30B). Consistently, the wt Env induced membrane exchange exclusively by fusion-mediated mechanisms, as it was blocked to the same extent by Leu3A and C34 (Figure 30B). These data suggested that fusion-independent trogocytosis does not occur from infected to uninfected T cells and hence may not explain the transfer of HIV particles across VSs. Besides, the lack of trogocytosis in the direction of viral transmission supports the idea that gag-driven HIV budding is necessary for viral transfer.

All together these results indicate that although CD4-dependent trogocytic processes occur during VSs, they appear to solely operate on the uninfected towards the infected cell direction.

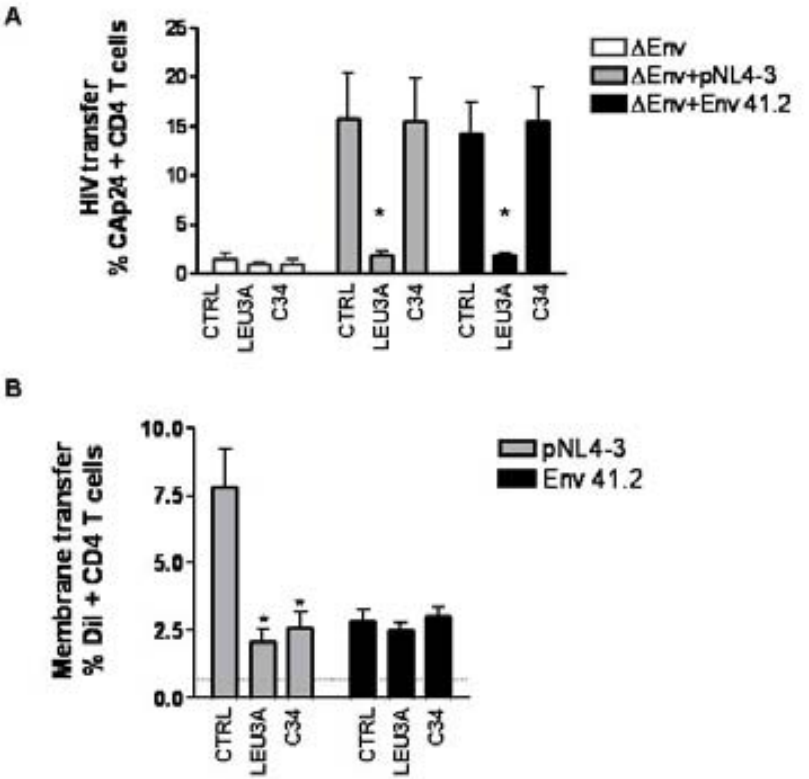

Figure 30. Analysis of trogocytosis on the HIV transmission direction. A. Cocultures of 293T cells transfected with an ΔEnv HIV backbone alone (white bars) or in combination with a wt Env(grey bars) or a fusion defective (41.2) Env that retains the ability to bind to CD4 (black bars) with primary CD4 T cells. Gag-dependent transfer was evaluated by CAp24 staining in the absence (Control) or the presence of Leu3A or C34. **B.** Trogocytic Gag-independent transfer of membranes was evaluated in cocultures of primary CD4 T cells with Env expressing cells stained with the lipophylic probe DiI. The wt NL4-3 Env was used as positive control of HIV transfer and fusogenic lipid transfer. Asterisks denote significant differences (* $p<0.025$, Bonferroni correction). Mean±SEM values of four different experiments are shown.

Chapter IV: *A Potential Outcome of Virological Synapses: Establishment of HIV Reservoir into Quiescent CD4+ T cells*

Activated CD4 T cells represent the major target for the virus to replicate although qCD4 have been shown to support low levels of HIV integration. Since HIV spread *in vivo* is enhanced by the formation of VS, we hypothesized that HIV transmission across VS may favor the formation of latent reservoirs. Thus, the dynamics of HIV infection in qCD4 T cells after direct T cell-T cell transmission have been evaluated in this chapter.

Primary isolated qCD4 T cells were cocultured with the chronically infected MOLT cells in the presence or absence of a transwell system to evaluate the impact of cell-free and cell-to-cell HIV infection, respectively. Then, total and episomal HIV DNA were quantified by real-time qPCR to determine HIV DNA synthesis and nuclear import respectively. Cell-to-cell HIV transmission was about 10 times more efficient in the establishment of infection into qCD4 T cells as compared to the cell-free virus model. Despite integration, the percentage of qCD4 T cells undergoing productive infection was under detectable levels as compared to activated CD4 T cells. Interestingly, Env-CD4 contacts induced phosphorylation of p56Lck and ZAP-70 into qCD4 T cells, although this stimulation was not sufficient to promote proliferative signals.

Therefore, that cell-to-cell HIV transmission may trigger viral latency in qCD4 T cells contributing to the establishment and continuous replenishment of long-lived HIV reservoirs. The contribution of VS-mediated infections should be considered in HIV eradication strategies.

4.1 Cell-to-cell HIV transmission enhanced infection of qCD4 T cells and reached the nucleus of target cells.

Following the model of T cell-T cell HIV transmission using infected MOLT cells and primary qCD4 T cells, the impact of VS in the generation of latently infected qCD4 T cells was analyzed by real-time qPCR. Cocultures of effector MOLT and qCD4 T cells or cultures of these cells separated by a transwell system were incubated for 24 hours to allow the virus to enter the target cell and reach the nucleus. Then, cocultures were pelleted to extract their DNA content and perform a serial of qPCR assays to quantify the total amount of HIV synthesis and the non-integrated episomal 2-LTR HIV form. The DNA copies of the endogenous CCR5 gene were monitored as a control of the number of cell present in each sample. In order to quantify the HIV infection solely in the qCD4 T cells present in the coculture system, the HIV DNA coming from MOLT cells was subtracted. To do so, a coculture stopped at time zero was employed. Results depicted in Figure 31A showed a ten times increase in the amount of HIV DNA synthesis from qCD4 T cells cocultured with $MOLT_{NL4-3}$ or $MOLT_{BAL}$ cells as compared to the cell-free viral infection. Addition of the anti-CD4 mAb Leu3A and the fusion inhibitor peptide C34 completely abrogated the infection to the same extend as in cocultures at time zero while the integrase inhibitor Raltegravir (RAL) did not have an effect on the amount of newly HIV synthesized (Figure 31B). These results showed that upon cellular contact, HIV transmission occurs more efficiently into

qCD4 T cells as compared with cell-free infections. The analysis of HIV DNA synthesis does not require HIV to enter the nucleus of the target cell as retrotranscription of the HIV RNA into DNA occurs in the cytoplasm. In order to resolve whether the proviral DNA can reach the nucleus of qCD4 T, measurement of the nuclear episomal 2-LTR form was performed. When the pre-integration complex of HIV enters the nucleus it can either remain as unintegrated DNA, or rather recombine and integrate into the host cell genome (provirus). Determination of the episomal 2-LTR form has been lately used as an indicator of recent infection events [4]. Therefore two sets of primers that bind to the joining region between the 2-LTR were used to quantify de novo infection into qCD4 T cells as compared to the time zero control (exhibiting the background of 2-LTR present in the MOLT cells). Addition of Raltegravir increased the formation of the 2-LTR circles as compared to the control coculture indicating the inhibition of DNA integration (Figure 31B). These results were confirmed in the coculture system as well as for cell-free virus infection (Figure 31B). All together, these data suggested that cell-to-cell HIV transmission enhances the infection rate of qCD4 T cells *in vitro*, where HIV is able to reach the nucleus of the target cell and integrate.

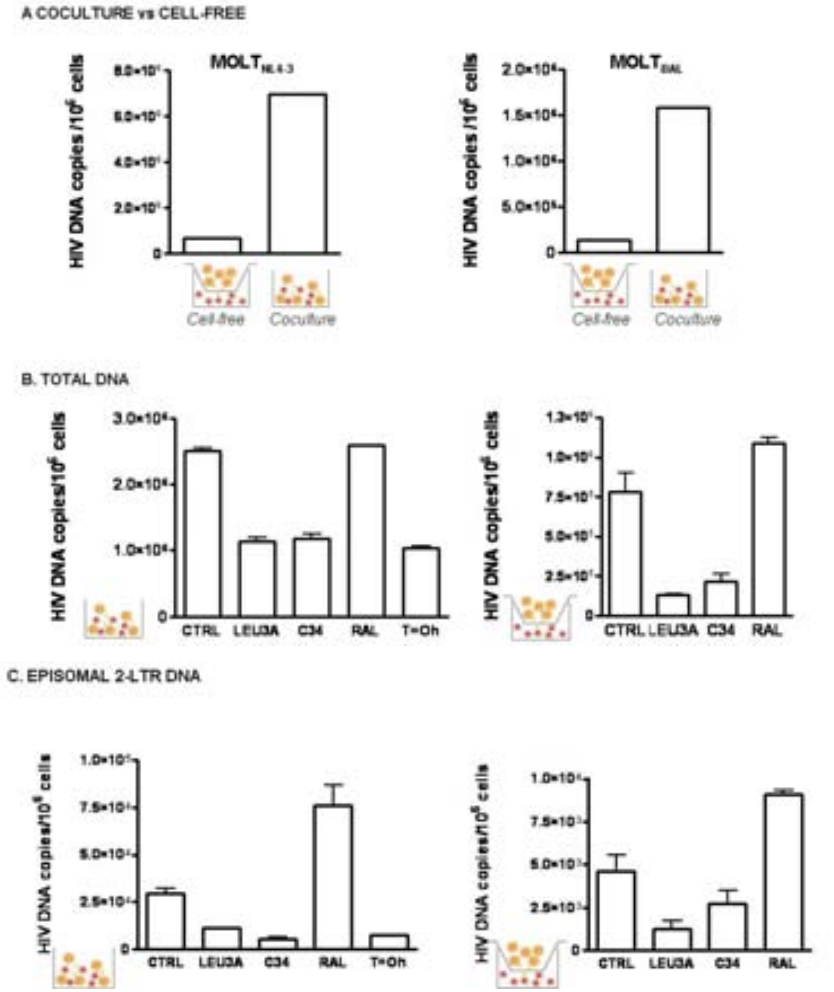
A COCULTURE vs CELL-FREE
HIV DNA copies /10^6 cells
MOLT NL4-3
MOLT BAL
Cell-free
Coculture
B. TOTAL DNA
HIV DNA copies/10^6 cells
CTRL
LEU3A
C34
RAL
T=0h
C. EPISOMAL 2-LTR DNA

Figure 31

Figure 31. HIV infection of qCD4 T cells is enhanced upon VS. A. Cocultures of primary qCD4 T cells with $MOLT_{NL4-3}$ or $MOLT_{BaL}$ cells were performed. After 24 hours DNA was extracted and the number of HIV copies per cell quantified using a qPCR system. HIV DNA content into MOLT cells was subtracted from the coculture sample using a coculture stopped at time zero. Values indicate the increase of HIV infection in cocultures as compared to cell-free infections. **B.** Newly synthesized HIV DNA was measured again in cocultures and cell-free infections (transwell) using $MOLT_{NL4-3}$ cells as effectors. **C.** Quantification of the episomal 2-LTR form was also assessed in the same cocultures and cell-free infection systems. Inhibitors tested were the blocking anti-CD4 mAb Leu3A, the fusion inhibitor peptide C34 and the integrase inhibitor Raltegravir. Cocultures stopped at time zero were indicative of the total and 2-LTR HIV DNA content inside the MOLT cells.

4.2 HIV DNA entered into the nucleus of qCD4 T cells but it did not trigger a productive infection

After having determined nuclear import of HIV DNA into qCD4 T cells cocultured with infected MOLT cells, the next step was to evaluate the transcriptional capacity of this resting CD4 T cell population containing the integrated provirus. By engineering genetic fusion proteins that are fluorescently tagged upon synthesis we can locate and track the transmission of HIV particles from infected to uninfected cells and the synthesis of new HIV particles as a measure of productive infection. 293T cells were cotransfected with an ΔEnv plasmid that contains a GFP reporter gene with an Env-coding plasmid to generate one-round replication pseudo-viruses. Transfection with the ΔEnv plasmid alone was used as a negative control of infection. Then, transfected cells were cocultured either with primary qCD4 T cells, PHA-activated CD4 T cells or the MT-4 cell line. Tracking the GFP expression by flow cytometry into the target cells determines HIV integration and subsequent transcription and translation into viral proteins. After 48 hours, cocultures were fixed and analyzed by flow cytometry. Infection of PHA-activated CD4 T cells and MT-4 cells was observed at 48 hours and abrogated in the presence of Leu3A or using 293T cells devoid of Env (ΔEnv) as effector cells. However, in qCD4 T cells, transcription of the integrated HIV provirus was not detected (Figure 32). These results indicated that even though qCD4 T cells can get infected, they do not produce viral particles suggesting that

the resting phenotype is hampering the steps following proviral integration.

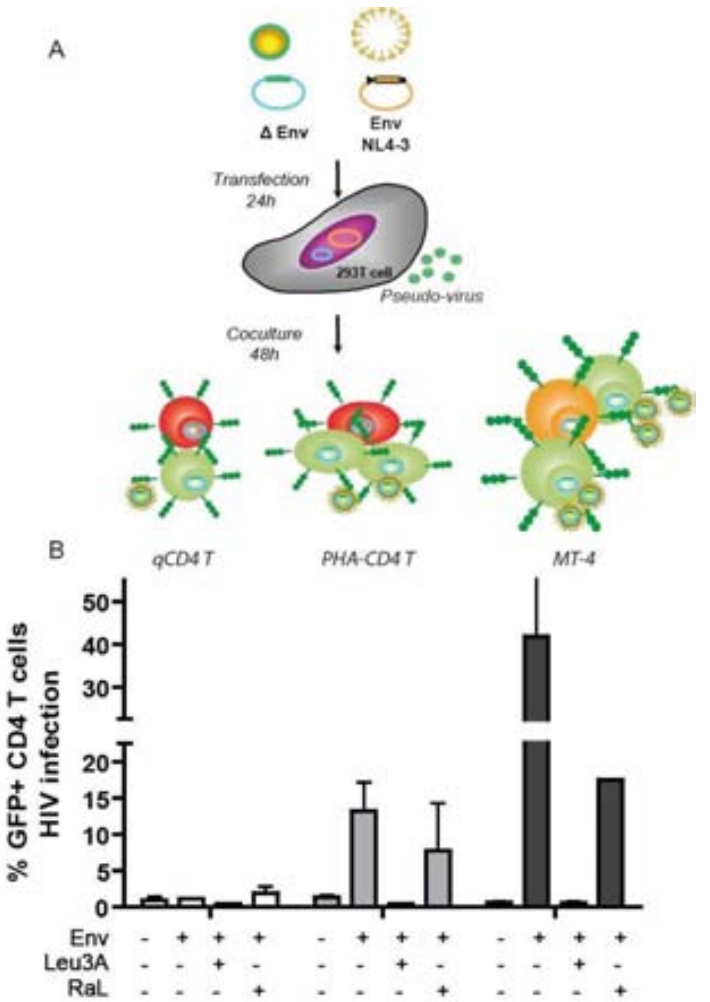

Figure 32

Figure 32. Cell-to-cell HIV infection of qCD4 T cells does not lead to productive infection. A. Schematic view of the experimental procedure performed to analyze productive infection. **B.** HIV transfected 293T cells were used as effector cells and cocultured with qCD4 T cells, PHA-activated CD4 T cells or the MT-4 cell line at a ratio 1:1. After 48 hours, analysis of productive infection was performed into target cells tracking the percentage of GFP positive cells by flow cytometry. Addition of the anti-CD4 antibody Leu3A and the integrase inhibitor Raltegravir were used as inhibitors of HIV entry and integration, respectively. 293T cells transfected with the ΔEnv plasmid alone were used as negative controls of infection.

4.3 Cell-to-cell HIV transmission modestly modulates the activation state of qCD4 T cells

Previous results pointed to state that cell-to-cell HIV transmission can infect qCD4 T cells but does not lead to productive infection leaving qCD4 T cells latently infected. We next aimed to evaluate if cell-to-cell HIV Env-mediated contacts would alter the resting phenotype of qCD4 T cells triggering activating signaling into qCD4 T cells after VS engagement. In Barat *et a.l* [157], HIV was found to efficiently replicate in qCD4 T cells cocultured with autologous DC due to the stimulation produced by the costimulatory molecules present in the infectious synapse. To analyze our hypothesis, several experiments were performed to test the proliferation capacity and the intracellular staining of the phosphorylated forms of p56Lck and ZAP-70 of qCD4 T cells cocultured with infected MOLT cells:

4.3.1 *The Effect of VS on CD4 T cell proliferation*

To analyze proliferation of qCD4 T cells after having contacted infected T cells, two different strategies were performed. First, primary qCD4 T cells were labeled with the cell tracker CMFDA and cocultured with $MOLT_{NL4-3}$ cells. PHA-stimulated CD4 T cells were used as a positive control of proliferation. Cell division results in sequential halving of fluorescence and up to 8 divisions can be monitored before the fluorescence is decreased to the background florescence of unstained cells. Second, the fluorescent DNA intercalating agent propidium iodide (IP) was used to track the DNA content as a measure of the cell division state. In both cases, the percentage of cells that have undergone cell division was monitored by flow cytometry. As illustrated in Figure 33A, qCD4 T cells remain in a non-dividing state after 5 days of culture when incubated either with a transwell system (cell-free) or in coculture with $MOLT_{NL4-3}$ cells.

Quiescent CD4 T cells did not divide under any condition while PHA-activated CD4 T cells showed an increase in the IP fluorescence as a result of the new DNA synthesis (Figure 33B). Cocultures of qCD4 T cells with $MOLT_{NL4-3}$ cells were not sufficient to induce proliferation into qCD4 T as compared to PHA-stimulated cells when measured either by CMFDA or IP staining. All together these data suggested that Env-CD4-mediated VSs are not sufficient to trigger proliferative signaling into qCD4 T cells, enabling the formation of latently infected CD4 T cells.

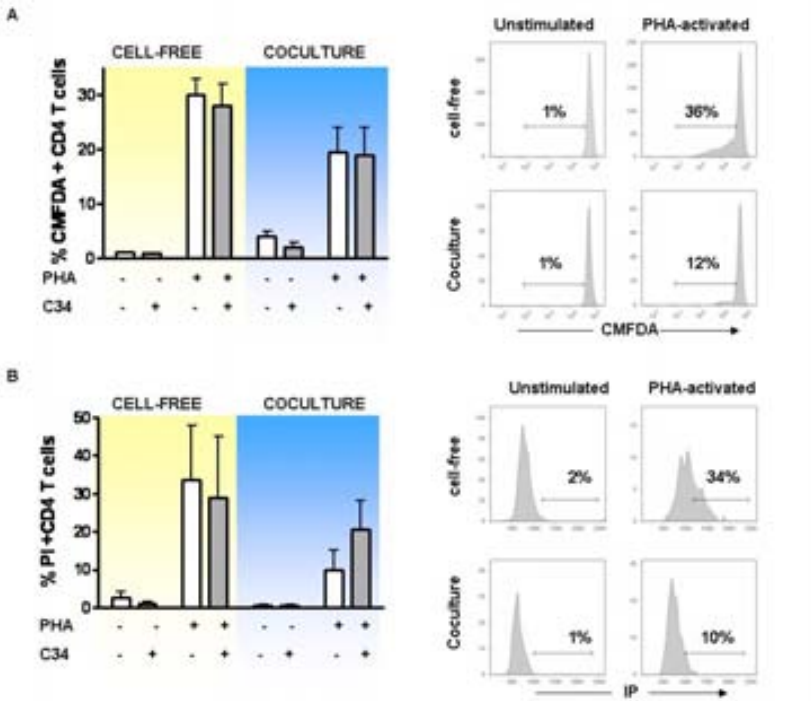

Figure 33. VS engagement does not induce proliferative signaling into qCD4 T cells. A. $MOLT_{NL4-3}$ cells were either cocultured or cultured in a transwell system (cell free) with CMFDA-labeled qCD4 T cells. Cocultures were left 3 days prior to analysis by flow cytometry. The percentage of proliferating cells is represented in the graph as those cells that have lost the initial CMFDA staining. One example of the analysis is illustrated at the right histograms. **B.** The same cocultures performed in A were stained for IP and analyzed also after 3 days of culture. The percentage of cells undergoing cell division is represented in the graph. One example of the analysis is illustrated at the right histograms. PHA-activated cells were used as positive controls of proliferation. The fusion inhibitor peptide C34 was added as a negative control of the gp41-mediated functions.

4.3.2 Effect of VS on p56Lck and ZAP-70 signaling

P56Lck is a tyrosine kinase associated to the cytoplasmatic tail of CD4 that upon activation phosphorylates another kinase from the signaling cascade termed ZAP-70. Since CD4 is the main receptor for HIV and ZAP-70 deficient cells show a delay in cell-to-cell HIV transmission as compared to the wt ZAP-70 expressing cells [86], it was hypothesized that Env-CD4 interaction at the VS could trigger the phosphorylation of p56Lck and ZAP-70 intracellular proteins into qCD4 T cells. To analyze this, intracellular staining of the p56Lck and ZAP-70 phosphorylated forms was performed following the manufacturer's instruction of the PhosFlow kit (BD, Bioscience) using antibodies against the phosphorylated forms of the two protein kinases. CD4 T cells coated with anti-CD3 and anti-CD28 mAbs following cross-link with a polyclonal IgG, expressed the higher amounts of the p56Lck and ZAP-70 phosphorylated proteins as compared to the unstimulated qCD4 T cells and were given the maximum levels of phosphorylation and used as positive controls for the experiments (Figure 34A). Cocultures of qCD4 T cells with either uninfected or $MOLT_{NL4-3}$ cells were left 30 minutes prior to initiate the staining protocol. Flow cytometry analysis showed a notorious increase in the MFI of ZAP-70 expressing cells cocultured with $MOLT_{NL4-3}$ cells as compared to the uninfected control or using the nAb IgGb12 against the Env CD4 binding site (Figure 34B). Levels of p56Lck expression in the same cocultures showed a slightly increase after VS engagement (Figure 34B).

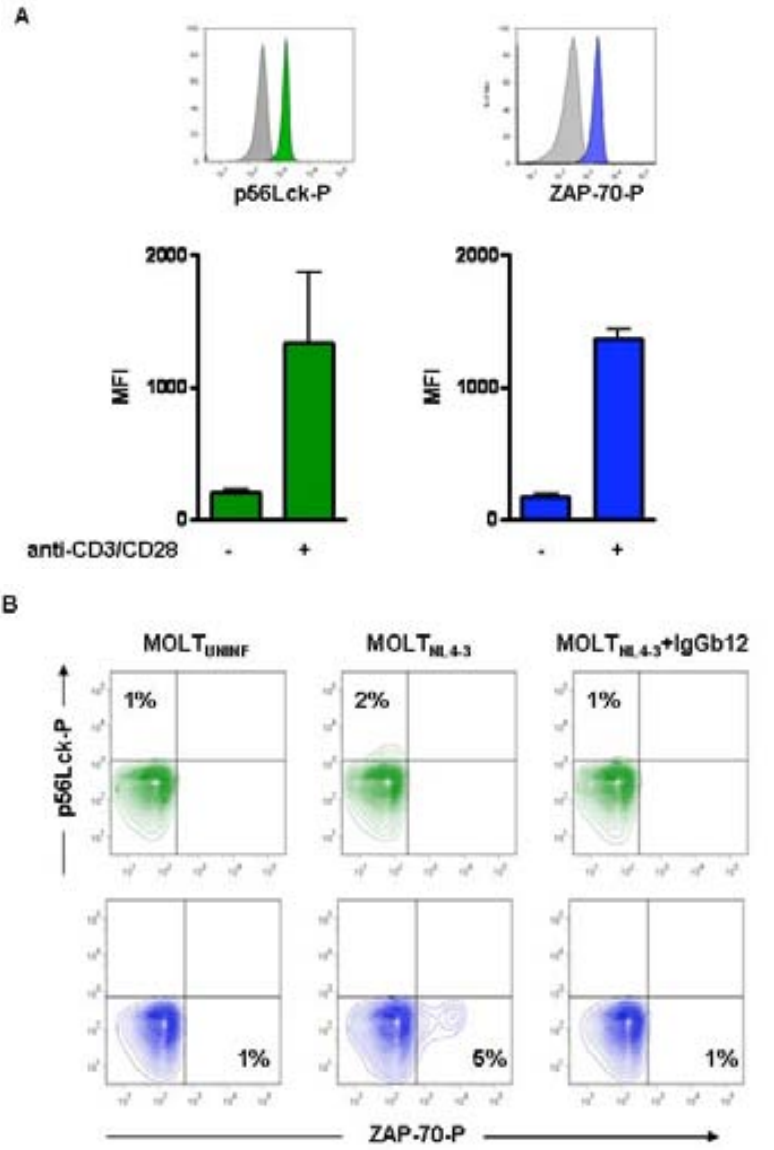
A
p56Lck-P
ZAP-70-P
2000
1000
0
MFI
anti-CD3/CD28
-
+
B
MOLT$_{UNINF}$
MOLT$_{NL4-3}$
MOLT$_{NL4-3}$+IgGb12
p56Lck-P
1%
2%
1%
1%
5%
1%
ZAP-70-P

Figure 34

Figure 34. Phosphorylation of p56lck and ZAP-70 is induced into qCD4 T cells after VS formation. A. Primary qCD4 T cells alone were stained for p56lck and ZAP-70 phosphorylated forms. CD4 T cells coated with anti-CD3 + anti-CD28 antibodies were used as positive controls phosphorylation. **B.** Cocultures of MOLTNL4-3 cells with qCD4 T cells were incubated for 30 min prior to staining. Cocultures with uninfected MOLT cells or addition of the anti-Env antibody IgGb12 were used as negative controls. The MFI values of p56lck and ZAP-70 staining are represented in the graph. Dot plots of p56lck and ZAP-70 staining are illustrated (right panels).

To enhance the effect on the expression of the phophorylated proteins, we increased the sensitivity of the assay by co-staining qCD4 T cells with the HIV CAp24 antigen and the phosphorylated p56Lck antibody. This scenario allowed us to identify the qCD4 T cells that have contacted with $MOLT_{NL4-3}$ cells (for their CAp24 content) and hence we increased the sensitivity of detection of the phosphorylated p56Lck. By gating into qCD4 T cells that stained positive for the CAp24 antigen, the expression of the p56Lck phosphorylated notably increased as compared to the qCD4 T cells that had not contacted $MOLT_{NL4-3}$ cells (i.e. CAp24 negative cells) (Figure 35A). Moreover, a kinetic analysis was performed at different time points of qCD4 T cells cocultured with $MOLT_{NL4-3}$ cells to determine the expression of p56Lck phosphorylated over time (Figure 35B). Initially, the expression levels of double positive CAp24-p56Lck qCD4 T cells increased until they reached a plateau (approximately 2h of coculture) followed by a decrease to almost the background levels observed with the CAp24 negative cells (Figure 35B).

The results in the expression of the signaling proteins p56Lck and ZAP-70 indicated that VS modulates the activation state of qCD4 T cells after having contacted infected T cells. These activation signals would be strong enough to likely contribute to HIV integration although not sufficient to initiate proliferation and productive infection.

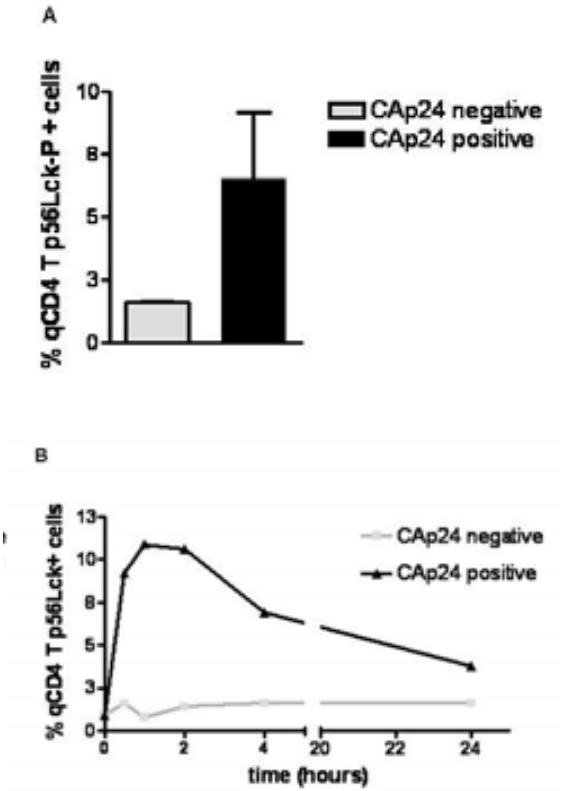

Figure 35. HIV-mediated cell-to-cell contact enhances phosphorylation of p56lck into qCD4 T cells with a fast kinetics. A. Cocultures of $MOLT_{NL4-3}$ cells with qCD4 T cells were incubated for 30 min and intracellular co-staining of the HIV CAp24 antigen and the p56lck phosphorylated performed. **B.**A kinetics of the coculture of $MOLT_{NL4-3}$ and qCD4 T cells was performed at different time points; 30 min, 1, 2, 4 and 24 hours. After each time point, co-staining of CAp24 and p56lck was conducted. Analysis of p56lck staining was done separately into the CAp24 positive and negative qCD4 T cell population.

DISCUSSION & PERSPECTIVES

To date, several approaches have been explored to determine the T cell-T cell HIV transmission mechanism(s). They mainly differ in the *in vitro* model used and the methodology employed to determine infection of target cells. All these results have yielded somewhat discordant results on how HIV can be transmitted between infected and uninfected T cells. We provide several lines of evidence that highlight the relevance of Env gp120 binding to CD4 as a primary determinant in the formation of T cell-T cell conjugates over the role of HIV coreceptors CXCR4 and CCR5 or the adhesion molecules studied in this thesis. Concerning coreceptors, it is well known that CXCR4 is highly expressed in cultured primary CD4 T cells, while CCR5 is restricted to a subset of memory cells [179]. Therefore, a potential active participation of the coreceptor in cellular conjugates should favor X4 Envs. However, our results demonstrated that both X4 and R5 Envs formed similar amounts of cellular conjugates with primary CD4 T cells. Furthermore, coreceptor blockade did not inhibit T cell-T cell contacts, which were only sensitive to CD4 or gp120 blockade. These results were in disagreement with data from Sattentau and colleagues, which have imaged the structure of VS by confocal microscopy and showed the recruitment of HIV receptors and coreceptors at the junction between infected and uninfected T cells [63] as observed by others in DC-T cell mediated synapses [50, 60]. Our results suggested that coreceptor, despite necessary for infection, does not have a relevant role in conjugate formation.

Then, we have observed how HIV exploits the cellular contacts and the resulting polarization of intracellular viral proteins to the cell-cell junction, to be massively transferred to uninfected cells. We proved that the Gag colocalization at the VS is exclusively dependent on Env-CD4 interaction using a ΔEnv virus [78] or blocking the Env-CD4 interaction with mAbs [180]. However, transfer of HIV across VS was unaffected by the addition of the fusion inhibitor peptide C34, neutralizing antiserum from infected patients [87, 181], coreceptor antagonists (AMD 3100 and JM-2987) or using a cell line defective for the appropriate coreceptor (MT-4 $CCR5^-$). Instead, the formation of Env-CD4 driven VS is sufficient to promote the massive transfer of HIV particles from infected to uninfected cells with a very rapid kinetics.

When analyzing data on cell-to-cell HIV spread, the target cell type used and the methodology employed to detect productive infection should be taken into account. The methodology that has been widely employed to characterize the cell-to-cell HIV spread mechanism(s) was the tracking of the HIV CAp24 protein by flow cytometry or microscopy [78, 87, 104, 181-183]. Moreover, we have also used an alternative approach to characterize transport phenomena at the VS by evaluating the transfer of membrane lipids, which are incorporated into newly released viruses upon budding. However, this could lead to misinterpretation of the data, since CAp24 or membrane labeling are techniques that on their own cannot discriminate between non-infectious and infectious transfer events and this fact should be kept in mind when analyzing data on cell-to-cell HIV spread.

Regarding the cellular type, MT-4 cells (and by extension other CD4 cell lines) due to their activated phenotype, may produce new viral particles after cell-to-cell transmission. Thus, tracking of HIV CAp24 or membrane lipids in these cells is sensitive to coreceptor blockade as it measures not only transfer but also productive infection. Conversely, the unstimulated phenotype of primary CD4 T cells hampers the rapid flow cytometric quantification of infection events, making these cells an excellent model to explore the mechanisms involved in early events of HIV transmission, namely HIV transfer. Thus we proposed that the simplest manner to overcome discrepant results on cell-to-cell HIV transmission analysis into primary T cells was to use an assay that measures outcomes directly relevant to infection. These include the use of qPCR to monitor the early synthesis of proviral DNA immediately after viruses have fused with target cells, or the access of HIV cores to the cytoplasm using fluorescent techniques [184] in cocultures with primary cells. Besides, when working with cell lines, other assays could also be employed; either using cell lines that activate a reporter construct upon HIV infection [78, 180] or the detection of de-novo synthesized, inhibitor-sensitive Gag production in the target cell using single cycle replication-competent pseudoviral vectors [45, 183, 185]. Our results showed that in the absence of coreceptor, cell-cell fusion and cell death are halted. Besides, productive infection of cell lines and HIV entry into primary cells is also inhibited. Thus, when coreceptor union is blocked, HIV transfer is the only consequence arising from the VS formation in a process

totally dependent on Env-CD4 interaction. Nonetheless, it remains to be elucidated whether the coreceptor-independent viral transfer across VSs may contribute to a final infection of target cells.

Taken together, our data suggest that when measuring membrane lipid transfer or HIV CAp24 transfer either by intracellular staining or using virus carrying the GFP-Gag fusion protein [78, 87, 180], we would be referring to the physical passage of HIV particles from infected to uninfected cells and thus we suggest to use the term "HIV transfer" whereas when this transfer leads to infection of the target cell, we suggest to keep the term "HIV transmission". Thus HIV particles would be transferred across synapses, while HIV infection would be transmitted between cells. Chronologically, HIV transfer is an early event occurring immediately after VS formation, which precedes but does not inevitably lead to transmission, a late event resulting in infection. It is thus very important to note these differences when analyzing the inhibition of cell-to-cell HIV transmission, in particular, the misinterpretation of HIV transfer as HIV transmission should be avoided.

Primary CD4 T cells present a unique ability to capture HIV particles into intracellular compartments in a fusion-independent manner a feature that may be explained by two non-exclusive mechanisms; first a massive budding of HIV particles that drag membrane components, followed by a massive binding and endocytic capture by uninfected target cells and /or second, trogocytic process involving the transfer of membrane patches carrying HIV budding machinery, a process that is probably linked to the formation of bridged structures (Figure 36). Of note, a new mechanism has recently been shown for HTLV-1 to transmit between cells independently of a synaptic contact; instead virions have been shown to bud at the plasma membrane where they are transiently kept in a meshwork of extracellular matrix (termed a 'viral biofilm') [186] reminiscent of the bacterial biofilms [187] yet it remains to be explored if this mechanism is also observed for other retroviruses such as HIV.

Cumulative evidence suggests that HIV transfer to primary cells follows the endocytic mechanism either by surfing along membrane bridges or as free viral particles released into the synaptic space. Electron microscopy data, from an early description [81] to a recent report by Chen *et al.* [78], described polarized Gag or Gag-GFP budding at the VS and endosomal vesicles containing HIV particles in target cells. Furthermore, biochemical data showed that transferred HIV materials are directed towards trypsin-resistant compartments [104], and videomicroscopy shows the vesicular

transfer of large amounts of Gag, sporadically involving entire synaptic buttons [78, 87, 104] which may lately result in a potentially productive infection [188]. Dynasore, a dynamin-dependent endosomal scission inhibitor, inhibited HIV replication supporting the model that endosomal fusion is associated with a productive pathway [188]. Endocytosis has been reported as an outcome of HIV transfer between infected and uninfected T cells either under fusion-permissive or fusion-nonpermissive conditions as colocalization of HIV proteins with markers of early endosomes has been observed [80]. In the absence of coreceptor, HIV transfer may be defined as a byproduct of HIV transmission that has been proposed to remain infectious for several days into nondegradative endosome or multivesicular body (MVB) [43, 104, 189, 190]. Bosch *et al* [80] propose that infectious virus could be "regurgitated" from recycling T cell endosomes into the extracellular space, in which it may go on to infect more cells, in a manner similar to the "Trojan horse" model proposed for DCs [61].

On the contrary, other authors have failed to image the endocytic uptake of HIV across VSs by confocal microscopy, electron microscopy or electron microscopy with tomography [63, 191]. The same authors hypothesize that as VS massively recruits viral budding into the synaptic junction, it may lead to the release of great amounts of immature HIV particles which would be easily taken up into endosomal compartments but would be inefficient to infect target cells. Therefore infection, according to their supposition, mainly comes from virus fusion at the plasma membrane level.

However, our results suggested us that the differences in cell-to-cell HIV transmission observed between primary CD4 T cells and a model cell line might be associated with the kinetics of hemifusion/fusion events; delayed fusion at the cell membrane may favor the duration of hemifusion, increasing endocytosis of HIV particles and cell death events in primary cells. In contrast, rapid fusion kinetics at the cell membrane may favor transmission of HIV infection with lower levels of endocytosis and gp41-mediated death in the cell line model.

Alternatively, trogocytic exchange of membrane patches, initially described during cellular contacts among cells of the immune system [106, 118], has been proposed as a possible mechanism for the transfer of HIV particles across the VS. Although trogocytosis has been associated with little cytoplasm transfer compared to plasma membrane transfer [121], this mechanism could be sufficient to allow viral proteins and genomic RNA, both polarized to synaptic areas [44], reach target cells (Figure 36). Of note, we observed trogocytosis at the VS before coreceptor engagement, potentially allowing for an open access of the HIV machinery to the cytoplasm of target cells. Our data showed trogocytosis of cell-surface proteins in the uninfected toward the infected cell direction but not on the HIV transmission direction. The unidirectionality of trogocytosis within HIV VSs together with the fact that coreceptor binding is not required to observe trogocytosis may be consistent with the reported elongation of CD4 T cell membranes (nanotubes or

filopodia) [111, 192] that are endocytosed by infected cells [111]. The lack of trogocytosis in the direction of viral transmission supports the idea that Gag-driven HIV budding is necessary for viral transfer and is also consistent with the electron microscopy images of VS [87]. Trogocytosis even though not participating actively in the transfer of HIV particles across the VSs, it may modulate the duration of the synapse according to its role in removing cell surface receptors [106, 120, 121].

Irrespective of the entry pathway preferred by HIV during cell-cell spread, fusion inhibitors and neutralizing antibodies have been shown by us and others to efficiently block the infectious process [106, 118]. Cell-cell HIV spread by DC-mediated trans-infection of primary CD4 T cells failed to block the infectious process when adding both nAbs [193, 194] or fusion inhibitors [195] suggesting that there is a component of DC-T cell viral transmission that is inaccessible to these types of drugs. Nonetheless, our model proposed is very consistent with the results obtained so far where T cell-T cell HIV transfer is a process that does not require coreceptor engagement and that may allow for a massive transfer of HIV particles into trypsin-resistant intracellular compartments. Thus, T cell-T cell HIV transmission is a later event resulting in target cell infection that only occurs upon Env-coreceptor recognition either at the cell membrane surface or at the endosomal membrane level (Figure 36).

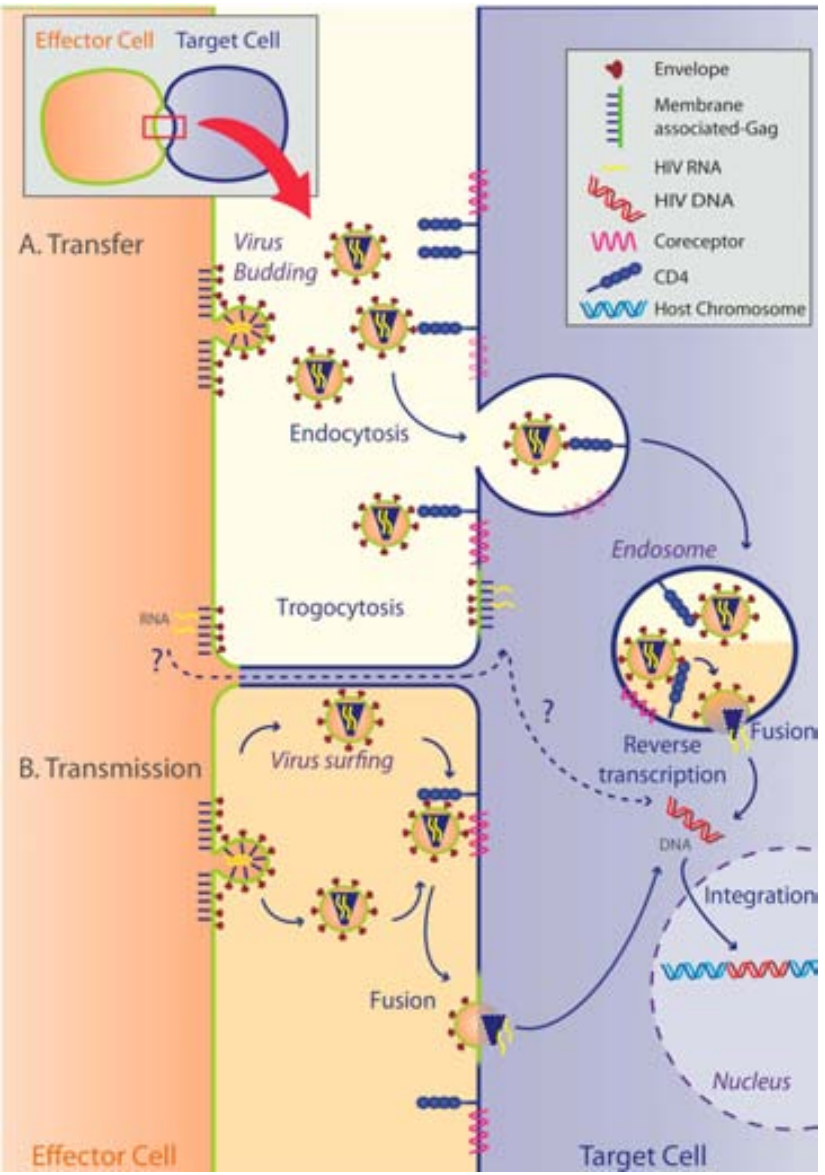
Effector Cell
Target Cell
Envelope
Membrane associated-Gag
HIV RNA
HIV DNA
Coreceptor
CD4
Host Chromosome
A. Transfer
Virus Budding
Endocytosis
Endosome
Trogocytosis
RNA
?
?
B. Transmission
Virus surfing
Reverse transcription
Fusion
DNA
Integration
Fusion
Nucleus
Effector Cell
Target Cell

Figure 56

Figure 36. Steps of HIV transmission. A portion of the VS (top left) has been enlarged to illustrate HIV transfer (A) and transmission (B). Two mechanisms would be involved in the transfer of HIV materials from infected to target cells after the VS formation. Both are dependent on Env binding to CD4 but independent of co-receptor engagement. First, a massive budding of viral particles from the infected cell (left) to the synaptic space (middle) and a further virion wrapping in the endosomal vesicles by the target cells (right). Second, membrane patches from infected cells carrying HIV budding machinery could be transferred to uninfected cells by trogocytosis through the formation of tethering tubes, potentially allowing for viral RNA to enter the cytoplasm of target cell (without exposure to the extracellular milieu). Furthermore, membrane tubes may help virions to surf extracellularly towards the uninfected cell. For cell-to cell transmission events (involving infection of target cells), viral particles require both CD4 and the co-receptor, CXCR4 or CCR5, to fuse with the target cell. This process may occur at the plasma membrane or in endosomal compartments, allowing for HIV RNA release into the cytoplasm and initiation of the infectious cycle, after reverse transcription and nuclear import. In the absence of the co-receptor, transferred HIV particles accumulate in the endosomal compartments.

While CD4 receptor and CXCR4 and CCR5 coreceptors are directly implied in the T cell-T cell HIV transmission, a large array of host cell-derived membrane proteins, are incorporated into the surface of HIV-1 such as LFA-1 and ICAM-1, which retain their biological functions when expressed on the virion surface, and have been shown to increase virus-cell interaction, enhance virus infectivity, and extend the host cell range of the virus [34, 89, 90, 196-201]. On the other hand, other membrane surface proteins known as tetraspanins, that are also recruited to sites of HIV budding, as evidenced by their incorporation into viral particles [202], have been shown to negatively modulate HIV dependent cell-to-cell fusion and transmission but have not been studied in here [95, 99]. Early observations show that incorporation of LFA-1 or ICAM-1 into HIV particles, notably increases the infection of target CD4 T cells [34, 89, 91] and at the same time it confers resistance to nAbs and to the fusion inhibitor T-20 [203, 204]. Although adhesion interactions influence HIV infection by serving as attachment cofactors for cell-free virions, their contribution to cell-cell dissemination has been little studied. Because LFA-1 is enriched at the VS [82], and in light of the importance of adhesion molecule interactions in both IS formation and cell-free HIV infection, we investigated the hypothesis that adhesion interactions promote and/or maintain T-cell VS formation and cell-cell HIV spread. We have observed that the potential role of LFA-1/ICAM-1 is unexpectedly secondary during T cell-T cell HIV transfer from MOLT infected cells to CD4 T cells, which is in disagreement with

previous findings [82]. Env-independent contacts between infected and uninfected T cells are near background levels and the blockade of adhesion molecules, employing inhibitory specific mAbs, did not significantly modify Env-mediated cellular conjugates or HIV transfer and transmission even though all antibodies were used at saturating concentration. Furthermore, in the absence of adhesion molecules (using 293T cells as effector cells), transfer of HIV to primary CD4 T cells still occurred, which would be consistent with early studies that reported cell-to-cell fusion and viral transfer into LFA-1 deficient cells or expressing low affinity forms of this integrin [92, 205]. However, a more recent report showed that morphological determinants of VSs appear to be inhibited in the absence of LFA-1/ICAM-1 but their role in cell-to-cell HIV spread was not characterized [82]. Controversially, our data were contradictory with the more relevant role reported in the infection of CD4 T cells by free HIV particles [34, 89, 91], and the complete requirement of these molecules for *trans*-infection induced by DCs [91-93, 206, 207]. Of note, T cell-T cell contacts and DC-T cell contacts are relatively different processes. In fact, for DC mediated *trans*-infection of CD4 T cells, HIV takes advantage of naturally occurring cellular conjugates during DC scanning of T cells or antigen presentation, a process where LFA-1 plays a relevant role in the strengthening and modulation of the IS. Thus, during DC-mediated HIV transmission, ICAM-1 on the DC and LFA-1 on the CD4 T cells partly mediate the *trans*-infectious process [93]. In contrast, for an efficient transmission between T cells, HIV forces the contact through Env expression on

the surface of infected cells, as T cell-T cell contacts are low in the absence of the HIV Env. It is very unlikely that T cell contacts may have a regulatory mechanism such as antigen presentation, which would be impaired due to Nef-induced down-regulation of MHC class II molecules and the fast kinetics of Env expression in infected cells [208, 209].

Nonetheless, our data point to a different role of ICAM-1 in HIV transfer from MOLT or 293T cells to uninfected CD4 T cells. We suggest that cellular conjugates and subsequent virus transmission are regulated by a balance between the expression of Env and adhesion molecules on effector cells. When HIV is presented by 293T cells, we detected additive contributions of both Env and ICAM-1 to HIV-1 transfer, most likely due to the higher expression of ICAM-1 as compared to Env. The ICAM-1 overexpression may increase the number of cellular conjugates or alternatively signal through LFA-1 in target cells. In contrast, during HIV presentation by infected CD4 T cells (primary or MOLT cells) the equilibrium of Env/ICAM-1 expression is completely unbalanced towards a full control of HIV transmission by Env binding to CD4.

Therefore, our results fully agree with a secondary role for adhesion molecules in the formation of cellular conjugates and the subsequent HIV transmission between infected and uninfected T cells. We, thus, demonstrated that the binding of Env to CD4 governs the formation of T cell-T cell contacts. Interestingly, LFA-1 and ICAM-3 appear to modulate the extent of HIV transfer between primary CD4 T cells, an effect that may be relevant for HIV spread *in vivo*.

On the other hand, by analogy to cell-free HIV attachment and entry, which preferentially targets memory CD4 T cells [37, 210, 211], we have also analyzed the selectivity of cell-to-cell HIV transfer to these two differential CD4 T cell subsets. Even though memory CD4 T cells expressed higher amounts of adhesion molecules, HIV transfer from MOLT, 293T and primary infected cells preferentially targeted $CD45RO^+$ CD4 T cells by a mechanism independent of LFA-1 and coreceptor expression. Several studies have reported the role of LFA-1 as a major determinant for this selectivity during cell-free viral infections [34, 37], while we have observed that in the absence of LFA-1, selectivity of cell-to-cell HIV transfer towards the memory subset is still observed [183]. A potential explanation could be associated with the organization of signaling molecules, including ZAP-70, which is more efficiently recruited to the IS in memory cells [212]. Besides, ZAP-70 signaling is also involved in the formation of VSs and cell-to-cell HIV spread [86]. On the other hand, a recent report suggested that this selectivity towards the memory subset could be associated to the fusogenic capacity of the X4 HIV Env to bind memory cells more efficiently than the naïve counterpart [213]. All in all, LFA-1-ICAM-1 interaction is not essential for HIV to spread across T cell-mediated VS and neither participates on the selectivity of HIV transfer towards the memory CD4 T cell subset.

From the early experiments of cell-to-cell HIV transmission to unstimulated primary CD4 T cells, we questioned the role of this highly efficient mode of HIV spread in the establishment of latently infected CD4 T cells. Our results in agreement with other studies [127, 129] showed integration of the provirus into quiescent CD4 T cells, a feature that was increased upon cell-to-cell contact as compared to cell-free viral infections. Moreover, we were able to detect integration by using an indirect assay that measures the unintegrated 2-LTR episomal HIV DNA form. When adding the integrase inhibitor Raltegravir, an increase in the 2-LTR form was observed whereas total HIV DNA levels were similar in the presence or absence of Raltegravir, suggesting that the integrated provirus was replaced by an increase of the episomal 2-LTR form. Despite integration, qCD4 T cells were unable to produce new viral particles as compared to previously activated CD4 T cells, consistently to previous data [128]. Env-coreceptor interaction promotes the cortical actin dynamics necessary for the establishment of HIV latent infection into resting T cells [161, 214]. Besides, a recent study has demonstrated that VSV-pseudotyped HIV particles are less efficient to enter the nucleus of resting T cells than the HIV itself, suggesting that a differential entry pathway is observed in VSV-pseudotyped viruses that is normally highly effective in transformed or metabolically active cells but defective in resting T cells [163]. However, our results showed that the Env-mediated signaling is not sufficient to promote HIV replication in qCD4 T cells. Therefore, our goal was to evaluate the signaling mechanism

that mediates HIV latent infection without new HIV particle production. To do so, we first analyzed the proliferative capacity of qCD4 T cells upon VS engagement and observed that the Env-mediated signaling is not sufficient to trigger proliferation, which is required for HIV propagation *in vitro*. Next, we have evaluated the role of p56Lck and ZAP-70 signaling molecules in VS. It has been widely described that upon TCR ligation, p56Lck which is bound to the cytoplasmatic region of CD4 [215], is activated and induces ZAP-70 recruitment to the IS (reviewed in [216]). This event initiates intracellular calcium mobilization, cytoskeletal reorganization and cell activation. However, the cellular proteins involved in the VS formation are not so well characterized. Our preliminary results likely indicate that after HIV cell-to-cell contact formation, p56Lck is phosphorylated, which suggested that Env-CD4 interaction is somehow activating the latently infected qCD4 T cells although this signaling is not sufficient to promote an efficient HIV replication. Our results correlate to previous reported data, which showed that ZAP-70 deficient cells were unable to support efficient expression of viral proteins and Gag colocalization at the VS, indicating a role of ZAP-70 in HIV entry and replication [86]. However, in VS contrary to IS, a F-actin depleted cSMAC was observed, which may provide an ideal route for efficient virus entry into the target CD4 T cells [83] but would not trigger the signaling required for cell activation and hence HIV replication. Recently, Thoulouze and others [217] showed that HIV-infected T cells exhibit impaired IS formation, and they attributed the defects to the effects of the HIV Nef protein.

Similarly, another study reported that Nef-associated activity inhibited actin polymerization and p56Lck recruitment to the IS [218]. Still further studies are needed to evaluate the role of intracellular proteins and their signaling effect during T cell-T cell HIV transmission. Irrespective of the signaling cascade associated with VS formation, our data clearly show that, *in vitro*, qCD4 T cells may integrate HIV DNA upon conjugation with infected cells but fail to produce new HIV particles, suggesting that VS may favor the formation and the replenishment of the HIV reservoirs *in vivo*.

The persistence of HIV integrated provirus in the genome of quiescent CD4 T cells from the peripheral blood and in tissues is one of the main challenges to underscore in the new era of HIV treatments. Several therapeutic approaches are aimed at eradicating the remaining HIV replication from those latently infected cells and at the same time maintaining or intensifying the treatment in order to prevent a new spreading infection. Some of these strategies involve reactivation of latent reservoirs, one of the most encouraging strategies that have lately been studied, which would help to rekindle the HIV infection present in the hidden reservoirs. New strategies are aimed at finding an ideal treatment that would reactivate latent HIV but avoid global T cell activation, a process that may induce viral replication and increase the number of susceptible uninfected target cells beyond the threshold that can be controlled by antiretroviral therapy. This is especially relevant after the recent demonstration that antiretroviral therapy may fail to

completely suppress HIV replication [148]. Thus, eradication strategies should consider a complete blockade of HIV replication, which at very low levels may occur in patients with undetectable levels of viremia. The role of cell-to-cell transmission in such a low level replication is an attractive hypothesis.

Yet many questions remain to be answered about cell-to-cell HIV transmission. Whether this mechanism is of relevance *in vivo*, today is still improbable, as all the research has been performed using *in vitro* experimental approaches. Additional investigations of cell-to-cell spread in ex vivo tissues and experimental models of nonhuman primate infection should shed light on the *in vivo* importance of this mode of spread. Even though VSs were born in the context of ISs, HIV seems to have evolved the ISs structure and functionality for their own spread ensuring its surveillance by avoiding T cell activation and remaining latent in cellular reservoirs. The current therapeutic approaches are not aimed at finally eradicating the disease. Antiretroviral treatment controls the viremia of infected patients; a feature that reverts when treatment is interrupted most probably due to the rebound of latently infected cells. The latter remain invisible for the immune system and reactivation using different therapeutic strategies is still not targeted to these cells and would lead to a global T cell activation state. Moreover, neutralizing antibodies against HIV are efficient to stop new cell-free HIV infections but their effectiveness during cell-to-cell spread has yet not been accomplished. Thus, characterization of

the molecular basis of HIV cell-to-cell spread is urgently needed to finally understand the viral pathogenesis and help design new therapeutic strategies aimed at specifically eradicate this highly efficient mode of spread. The new era of cell-to-cell HIV research should consider many facts; the *in vitro* culture model used should be taken into account when interpreting data, the necessity to test HIV treatments in the context of VSs spread, the fact that the different mechanisms of cellular intercommunication can be hijacked by HIV to spread throughout and the contribution of VSs in HIV infection *in vivo*. In summary, the formation, mechanisms and outcomes of virological synapses need further investigation in the upcoming years.

CONCLUSIONS

The main conclusions that came up from the studies presented in this thesis are summarized below:

1. The main driving force that directs the formation of the T cell-T cell VS is the interaction of the Env glycoprotein with its receptor CD4 in the target cell.

1.1 The blockade of the adhesion molecules ICAM-1, LFA-1 and ICAM-3 is not sufficient to inhibit neither cellular contacts nor T cell-T cell HIV transfer and transmission. Nevertheless, LFA-1 and ICAM-3 appear to modulate HIV transfer between primary cells.

1.2 Selectivity of HIV transfer towards memory CD4 T cells is independent of the higher expression of LFA-1 in memory as compared to naïve CD4 T cells.

2. The early events that take place soon after the VS formation are named HIV transfer and are independent of coreceptor engagement. This mechanism precedes, but does not necessarily leads to HIV transmission, a later event resulting in a fusion-dependent HIV infection.

2.1 Primary CD4 T cells are a suitable model to explore HIV transfer events due to their unstimulated phenotype and their ability to capture higher amounts of HIV particles into trypsin-resistant compartments.

2.2 On the other hand, cell lines are more suitable models to explore HIV transmission mechanisms due to their activated phenotype. In primary cells, this late event can be also monitored by the synthesis of proviral DNA.

3. Trogocytosis exclusively operates from the uninfected towards the infected cell but not in the HIV transmission direction so it does not explain the transfer of HIV particles across the VS.

4. Despite HIV integration into qCD4 T cells following VS formation, this mechanism of viral spread does not provide the necessary signaling to support a productive infection *in vitro* and hence promotes the establishment of latent HIV reservoirs.

4.1 An indirect assay that measures the unintegrated 2-LTR episomal HIV DNA form was employed that indirectly assessed HIV integration in cell-to-cell transmission.

4.2 A substantial increase in the levels of phophorylated p56Lck and ZAP70 expression were detected after cell-mediated Env-CD4 interaction, which was not linked to cell proliferation or HIV production.

PUBLISHED ARTICLES

1. Human immunodeficiency virus type 1 entry and infection of T lymphocytes require ADP-ribosilation factor 6-mediated membrane dynamics. García-Expósito L, Barroso-González J, Puigdomènech I, Machado J D, Blanco J and Valenzuela-Fernández A. Paper under revision

2. Could CD4 capture by CD8+ T cells play a role in HIV-1 spreading? Aucher A, Puigdomènech I, Joly E, Hudrisier D and Blanco J.
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