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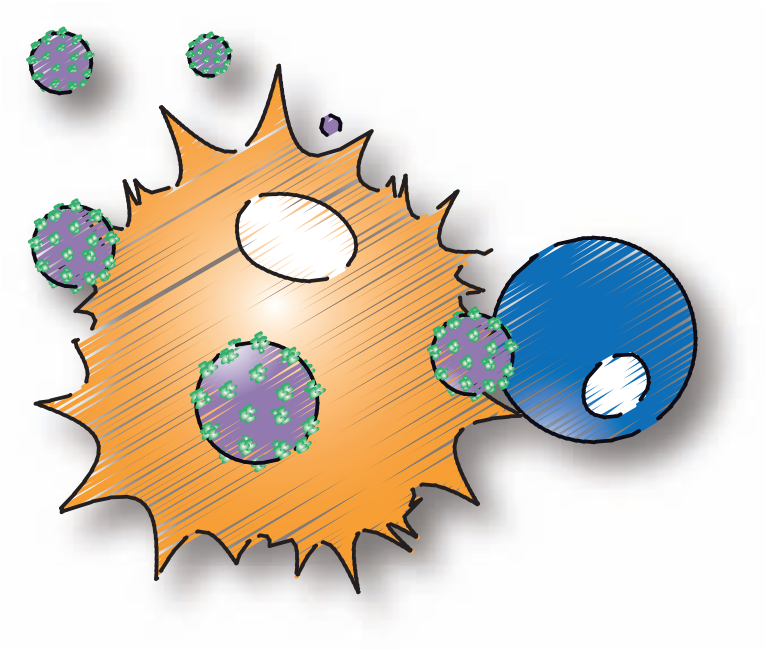

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# Dendritic Cells and HIV-1: Molecular Mechanisms Involved in Viral Capture, *Trans*-infection and Antigen Presentation

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Certifica:

Que el treball experimental i la redacció de la memòria de la Tesi Doctoral titulada "Dendritic Cells and HIV-1: molecular mechanisms involved in viral capture, *trans*-infection and antigen presentation" han estat realitzades per na Mª Teresa Rodríguez Plata sota la seva direcció i considera que és apta per ser presentada per optar al grau de Doctora en Immunologia per la Universitat Autònoma de Barcelona.

I per tal que quedi constància, signa aquest document.

Badalona, el 9 de maig de 2013.

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Badalona, el 9 de maig de 2013.

Dra. Dolores Jaraquemada Pérez de Guzmán

*A mis padres*

***"Muy floja y mediocre será la obra cuyo autor no haya empleado para tejerla fibras del corazón y hebras del cerebro."***

**Don Santiago Ramón y Cajal**
Premio Nobel de Medicina (1906)

# ABBREVIATIONS COMMONLY USED

| | |
|---|---|
| ABS | antibody binding sites |
| AIDS | acquired immunodeficiency syndrome |
| APC | antigen-presenting cells |
| APC | allophycocyanin *(flow cytometry)* |
| APC-Cy7 | allophycocyanin-Cy7 conjugate |
| AZT | azidothymidine |
| | |
| BlaM-Vpr | β-lactamase-Vpr chimera |
| | |
| CA | capsid |
| Cer | ceramide |
| CFDA-SE | carboxyfluorescein diacetate succinimidyl ester |
| CMRA | CellTracker™ Orange CMRA |
| CLR | C-type lectin receptors |
| CRF | circulating recombinant forms |
| cSMAC | central region of supra-molecular activation complexes |
| CTL | cytolytic $CD8^+$ T lymphocytes |
| | |
| DAPI | 4,6-diamidino-2-phenylindole |
| DC | dendritic cells |
| DC-SIGN | DC-specific ICAM-3–grabbing non-integrin |
| DDAO | CellTrace™ Far Red DDAO-SE |
| DMEM | Dulbecco's Modified Eagle medium |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| dsDNA | double-stranded deoxyribonucleic acid |
| dSMAC | distal supra-molecular activation complexes |
| dsRNA | double-stranded ribonucleic acid |
| | |
| eGFP | enhanced green fluorescent protein |
| ELISA | enzyme-linked immunosorbent assay |
| ELISPOT | enzyme-linked immunosorbent spot |
| Env | viral envelope glycoprotein |
| ESCRT | endosomal sorting complexes required for transport |

| | |
|---|---|
| FBS | fetal bovine serum |
| FITC | fluorescein |
| FRET | fluorescent resonance energy transfer |
| FSC | forward scatter chanel |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GSL | glycosphingolipid |
| GalCer | galactosylceramide |
| GM3 | 5-acetyl-alpha-neuraminic acid (2-3) beta-D-galactopyranose (1-4) beta-D-glucopyranose (1-1) ceramide |
| HAART | highly active antiretroviral therapy |
| HIV-1 | human immunodeficiency virus type 1 |
| HIV-2 | human immunodeficiency virus type 2 |
| HLA | human leukocyte antigen |
| ICAM-1 | intercellular adhesion molecule 1 |
| ICAM-3 | intercellular adhesion molecule |
| iDC | immature DC |
| iDC+ITIP | ITIP-matured DC during viral loading |
| iDC+LPS | LPS-matured DC during viral loading |
| IFN | interferon |
| IL-1 | interleukin-1 |
| IL-2 | interleukin-2 |
| IL-4 | interleukin-4 |
| IL-6 | interleukin-6 |
| ITIP | interleukin-1 β, tumor necrosis factor α, interleukin-6 and prostaglandin E2 |
| LC | Langerhan's cells |
| LFA-1 | leukocyte function–associated antigen |
| LPS | lipopolysaccharide |
| LTR | long terminal repeats |
| LUV | large unilamellar vesicles |
| MA | matrix |
| mAb | monoclonal antibody |
| mDC | mature dendritic cell |
| mDC ITIP | DC matured with ITIP for 48 hours |

| | |
|---|---|
| mDC LPS | DC matured with LPS for 48 hours |
| MDDC | monocyte-derived dendritic cells |
| MFI | geometric mean fluorescence intensity |
| MHC | major histocompatibility complex |
| MLR | mixed lymphocyte reaction |
| MLV | murine leukemia virus |
| MOI | multiplicity of infection |
| MPLA | monophosphoryl lipid A |
| mRNA | messenger RNA |
| myDC | myeloid dendritic cells |
| NC | nucleocapsid |
| NK | natural killer cells |
| NKT | natural killer T cells |
| NLR | nucleotide-binding oligomerization domain (NOD)-like receptors |
| NNRTI | non-nucleoside reverse transcriptase inhibitors |
| NRTI | nucleoside reverse transcriptase inhibitors |
| NtRTI | nucleotide reverse transcriptase inhibitors |
| NVP | nevirapine |
| PAMP | pathogen-associated molecular patterns |
| PBMC | peripheral blood mononuclear cells |
| PCR | polymerase chain reaction |
| pDC | plasmacytoid dendritic cells |
| PE | phycoerythrin |
| PE-Cy5 | phycoerythrin-Cy5 conjugate |
| PerCP | peridinin chlorophyll protein |
| PerCP-Cy5.5 | peridinin chlorophyll protein-Cy5.5 conjugate |
| PGE2 | prostaglandin E2 |
| PHA | phytohemagglutinin |
| pMHC | peptide–major histocompatibility complex |
| polyI:C | polyinoisic:polycytidylic acid |
| PRR | pattern-recognition receptors |
| PS | phosphatidylserine |
| pSMAC | peripheral supra-molecular activation complexes |
| qRT-PCR | quantitative real-time PCR |

| | |
|---|---|
| RLR | retinoic acid-inducible gene I (RIG I)-like receptors |
| RNA | ribonucleic acid |
| RPMI | Roswell Park Memorial Institute medium |
| SEA | superantigen; staphylococcal enterotoxin A from *Staphylococcus aureus* |
| SFV | simian foamy virus |
| Siglec-1 | sialic acid-binding Ig-like lectin 1 |
| SIV | simian immunodeficiency virus |
| SMAC | supra-molecular activation complexes |
| SP1 | spacer peptide 1 |
| SP2 | spacer peptide 2 |
| SQV | saquinavir |
| SSC | side scatter chanel |
| ssRNA | single-stranded ribonucleic acid |
| TcR | T-cell receptor |
| TGF-β | transforming growth factor β |
| $T_H$ | $CD4^+$ T helper cells |
| TLR | toll-like receptors |
| TNF-α | tumor necrosis factor α |
| tRed | texas red |
| VLP | virus-like particles |
| VSV | vesicular stomatitis virus |

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Dendritic cells (DC) are the most potent antigen-presenting cells in the immune system, linking innate and adaptive immune responses. However, it has been suggested a dual role of DC in Human Immunodeficiency Virus type 1 (HIV-1) infection by increasing the spread of HIV-1 while trying to trigger an adaptive response against viral infection. The classical immunological paradigm affirms that immature DC mainly mediate pathogen uptake while mature DC launch adaptive immune responses against the captured pathogen. Nevertheless, DC maturation with lipopolysaccharide (LPS) increases their ability of capturing HIV-1 particles resulting in a potent infectious transmission to target cells. The aim of this thesis is to analyze the mechanisms involved in DC-mediated HIV-1 capture, *trans*-infection and antigen presentation. To that end, we used methods of cellular and molecular biology, reporting the viral ligand and the DC receptor responsible for an HIV-1 Env-independent uptake mechanism, the fate of the captured HIV-1 particles, and the determinants for an efficient infectious transmission to $CD4^+$ T lymphocytes.

Our results provide new insights into contribution of DC to HIV-1 pathogenesis. Maturation of DC with LPS increases the expression of Siglec-1 on DC membrane. This receptor acts as the attachment factor in DC for sialyllactose-containing gangliosides in HIV-1 membrane, mediating the Env-independent mechanism of HIV-1 binding and uptake by DC. However, this efficient mechanism of HIV-1 capture does not represent a source of viral antigen for HLA loading and T-cell activation, given that captured virions are retained within an intracellular compartment, away from degradation, thus preserving their infectivity. Consequently, in the case of HIV-1 and DC matured with LPS, there is dissociation between pathogen uptake and antigen presentation. On the contrary, Siglec-1-mediated HIV-1 capture by DC is redirected to infectious viral transmission to susceptible target cells, without infecting host DC. Since DC continuously interact with $CD4^+$ T lymphocytes during their immunological labor, either to elicit adaptive immune responses or to maintain T-cell homeostasis, HIV-1 can take advantage of these contacts, without modulating them, to be transmitted. As a result, HIV-1 can go unnoticed across the DC–T-cell synapses, bypassing the immunological control and increasing viral dissemination, which is markedly favored by immune activation driven by DC–T-cell contacts. Therefore, determining the contribution of DC to the pathogenesis of HIV-1 infection may be important for the development of therapeutic strategies aiming to block HIV-1 spread.

Les cèl·lules dendrítiques (CD) són les cèl·lules presentadores d'antigen més potents del sistema immuntari, enllaçant la immunitat innata i la immunitat adquirida. No obstant això, les CD poden contribuir a la disseminació del Virus de la Immunodeficiència Humana tipus 1 (VIH-1) mentre intenten induir una resposta adaptativa contra la infecció viral. El paradigma immunològic clàssic sobre la funció de les CD indica que les CD immadures principalment capturen patògens, mentre que les CD madures indueixen una resposta adaptativa contra el patogen capturat. Però, la maduració de les CD amb lipopolisacàrid (LPS) augmenta la seva abilitat per capturar el VIH-1, que, al seu torn, resulta en una potent transmissió infecciosa a cèl·lules diana. L'objectiu d'aquesta tesi és el d'analitzar els mecanismes involucrats en la captura, la *trans*-infecció i la presentació antigènica del VIH-1 per part de las CD. Amb aquesta finalitat i utilitzant tècniques de biologia cel·lular i molecular, s'ha identificat el lligand viral i el receptor en les CD responsable del mecanisme de captura del VIH-1 independent de l'envolta viral, el destí del VIH-1 capturat per les CD, i els determinants per a una transmissió infecciosa eficient d'aquestes partícules virals als limfòcits T $CD4^+$.

Els resultats obtinguts proporcionen nova informació sobre la contribució de les CD a la patogènesi del VIH-1. La maduració de les CD amb LPS augmenta l'expressió de Siglec-1 a la membrana plasmàtica de les CD. Aquest receptor actua reconeixen la sialillactosa dels gangliòsids de la membrana del VIH-1, constituint el mecanisme d'unió i captura del VIH-1 en la CD independent de l'envolta viral. Però, aquest mecanisme tant eficient de captura viral en les CD no representa una font d'antigen per a la presentació antigènica del VIH-1, ja que les partícules virals capturades són retingudes en un compatiment intracel·lular, protegides de la degradació i preservant la seva infectivitat. Per tant, en el cas de les CD madurades amb LPS, hi ha una dissociació entre la captura del VIH-1 i la seva presentació antigènica. Per contra, la captura del VIH-1 per Siglec-1 es redirigeix cap la transmissió infecciosa a cèl·lules diana, però sense que el VIH-1 infecti les CD. Atès que les CD interaccionen contínuament amb les cèl·lules T $CD4^+$ durant la seva tasca immunològica, ja sigui per induir respostes immunes adaptatives o per a mantenir l'homeostasi de les cèl·lules T, el VIH-1 pot aprofitar-se d'aquests contactes, sense modular-los, per a ser transmès. D'aquesta manera, el VIH-1 passa desapercebut a través de la sinapsi CD–cèl·lula T, escapant del control immunològic i incrementant la propagació viral, que està marcadament afavorida per l'activació immune induïda pels contactes CD–cèl·lula T. Per tant, determinar la contribució de les CD en la patogènesi de la infecció pel VIH-1

pot ser de gran importància pel desenvolupament d'estrategies terapèutiques dirigides a bloquejar la disseminació del VIH-1.

Las células dendrítcas (CD) son las células presentadoras de antígeno más potentes del sistema inmunitario, enlazando la inmunidad innata y la inmunidad adquirida. Sin embargo, las CD pueden contribuir a la diseminación del Virus de la Inmunodeficiencia Humana tipo 1 (VIH-1) mientras intentan inducir una respuesta adaptativa contra la infección viral. El paradigma inmunológico clásico sobre la función de las CD indica que las CD inmaduras principalmente capturan patógenos, mientras que las CD maduras inducen una respuesta adaptativa frente al patógeno capturado. No obstante, la maduración de las CD con lipopolisacárido (LPS) incrementa su abilidad para capturar VIH-1, que, a su vez, resulta en una potente transmisión infecciosa a células diana. El objetivo de esta tesis es el de analizar los mecanismos involucrados en la captura, la *trans*-infección y la presentación antigénica del VIH-1 por parte de las CD. Con esta finalidad y utilizando técnicas de biología celular y molecular, se ha identificado el ligando viral y el receptor en las CD responsables del mecanismo de captura del VIH-1 independiente de la envuelta viral, el destino del VIH-1 capturado por las CD, y los determinantes para una transmisión infecciosa eficiente de estas partículas virales a los linfocitos T $CD4^+$.

Los resultados obtenidos proporcionan nueva información acerca de la contribución de las CD en la patogénesis del VIH-1. La maduración de las CD con LPS incrementa la expresión de Siglec-1 en la membrana plasmática de las CD. Este receptor reconoce la sialillactosa de los gangliósido de la membrana del VIH-1, mediando el mecanismo de unión y captura del VIH-1 en las CD independiente de la envuelta viral. Sin embargo, este mecanismo tan eficiente de captura viral en las CD no representa una fuente de antigéno para la presentación antigénica del VIH-1, dado que las partículas virales capturas son retenidas en un compartimento intracelular, protegidas de la degradación y preservando su infectividad. Por consiguiente, en el caso de las CD maduradas con LPS, existe una disociación entre la captura del VIH-1 y su presentación antigénica. Por el contrario, la captura de VIH-1 mediada por Siglec-1 se redirige hacia la transmisión infecciosa a células diana, pero sin que el VIH-1 infecte las CD. Dado que las CD interaccionan continuamente con los célula T $CD4^+$ durante su labor inmunológica, tanto para inducir respuestas inmunes adaptativas como para mantener la homeostasis de las células T, el VIH-1 puede aprovecharse de estos contactos, sin modularlos, para ser transmitido. De esta manera, el VIH-1 pasa desapercibido a través de la sinapsis CD–célula T, escapando del control inmunológico e incrementando la propagación viral, que está marcadamente favorecida por la activación immune inducida por los contactos CD–célula T. Por lo

tanto, determinar la contribución de las CD en la patogénesis de la infección por VIH-1 puede ser de gran importancia para el desarrollo de estrategias terapéuticas dirigidas para bloquear la diseminación del VIH-1.

# Chapter 1

# INTRODUCTION

# 1. Human Immunodeficiency Virus type 1

## 1.1 History

In June 1981, clinicians from Los Angeles (United States of America) reported the first cases of Acquired Immunodeficiency Syndrome (AIDS) in homosexual men with *Pneumocystis carinii* pneumonia and mucosal candidiasis [1]. Patients had no records of disease, but all presented a depletion of $CD4^+$ T lymphocytes and unusual infections and cancers, such as Kaposi's sarcoma. It was not until 1983 when a new human retrovirus, at the time known as lymphoadenopathy-associated virus (LAV), was isolated from a lymph node biopsy of an AIDS patient and identified as the etiological agent causing the disease [2]. In 1984, two other research groups further confirmed the novel retrovirus [3, 4], which was finally designated as Human Immunodeficiency Virus (HIV) by the International Committee on the Taxonomy of Viruses [5]. In 1986, a distinct retrovirus was isolated from West African patients with AIDS, and was named HIV type 2 (HIV-2) [6] and the original virus isolates HIV-1.

Current evidence indicates that HIV viruses entered the human population in the 1930s [7, 8] through multiple zoonotic infections from simian immunodeficiency virus (SIV)-infected nonhuman primates [9]. Specifically, HIV-1 is closely related to SIVcpz isolated from the chimpanzee subspecies *Pan troglodytes troglodytes* [10], whereas HIV-2 is highly associated with SIVsm from sooty mangabeys *Cercocebus atys* [11]. Nowadays, HIV/AIDS remains one of the most serious global health problems because of its high morbidity and mortality rates. In 2011, it was estimated that around 34 people were living with HIV, with the 69% of infected people residing in Sub-Saharan Africa. In the same year, 2.5 million people got newly infected with HIV while 1.7 million people died of AIDS-related causes [12]. Nevertheless, more people than ever are living with HIV, largely due to greater access to antiretroviral therapy.

## 1.2 HIV classification

The Human Immunodeficiency Virus (HIV) belongs to the group VI of reverse transcribing viruses, *Retroviridae* family, *Orthoretrovirinae* subfamily, *Lentivirus* genus, and includes the HIV-1 and HIV-2 species [13]. HIV-1 and HIV-2 species genetically diverge by at least 40%. Both viruses are similar in many ways, but their replicative and pathogenic capacity, virus evolution and target of infection may differ from each other. The most prevalent virus is HIV-1, which is spread all over the world. On the contrary, HIV-2 is confined to West Africa mainly because of its poor capacity for transmission,

low virus load and less pathogenic course of infection [14]. HIV-1 is classified into three major groups: M (*major*), O (*outlier*) and N (*new*). M group accounts for the majority of infections and can be divided into clades, which vary by 15–20%: A, B, C, D, F, G, H, J, K and Circulating Recombinant Forms (CRF) [15].

### 1.3 HIV-1 genome

The HIV-1 genome is composed of two positive single-stranded RNA (ssRNA) copies of approximately 9.8 kb in length, flanked by a repeated sequence known as the long terminal repeats (LTR) [16]. The viral genome codifies for nine genes that enable HIV-1 to be integrated into the host genome and use the cell's machinery to generate new viruses. Besides having three major genes coding for structural proteins (*gag*, *pol* and *env*), common to all retroviruses, HIV-1 has also six unique additional genes, with regulatory (*tat* and *rev*) or accessory (*vif*, *vpr*, *vpu* and *nef*) functions (Fig. 1).

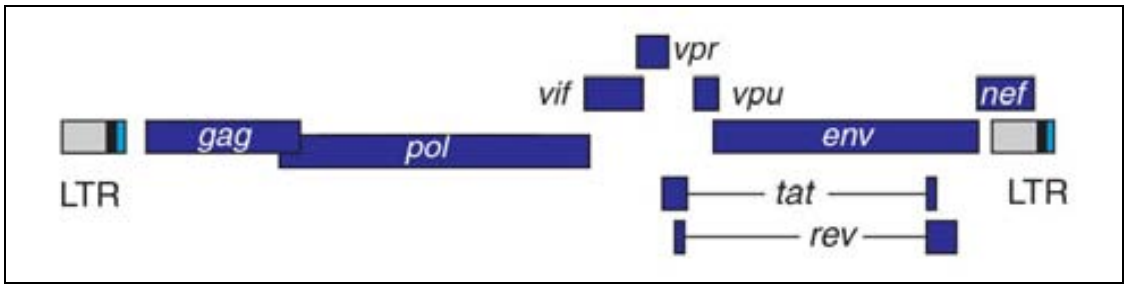


**Figure 1.** Schematic diagram of HIV-1 genome showing gene names in boxes. *Adapted from Kern, J. and Stoltzfus, C.M., 2012* [17].

- *gag*: codes for the Gag polyprotein, which is processed during maturation to generate the structural proteins p17 matrix (MA); p24 capsid (CA); p7 nucleocapsid (NC); p6; and two spacer peptides, p2 spacer peptide 1 (SP1) and p1 spacer peptide 2 (SP2).
- *pol*: codes for viral enzymes Reverse Transcriptase, Integrase, and Protease.
- *env*: codes for gp160, the precursor of gp120 and gp41 proteins, that are embedded in the viral envelope and enable the virus to attach to and fuse with target cells.
- *tat* and *rev*: code for regulatory proteins Tat and Rev, which regulate the transcriptional and posttranscriptional HIV-1 gene expression.

- *vif*, *nef*, *vpr* and *vpu*: code for regulatory proteins Vif, Nef, Vpr and Vpu, respectively; although they are initially dispensable for infection, they are important for efficient infection *in vivo*.

### 1.4 HIV-1 morphology

HIV-1 mature virions are spherical particles of 100-150 nm in diameter and wrapped by a lipid bilayer derived from the host cell membrane (Fig. 2). Viral spikes consisting of trimers of surface glycoprotein gp120 and transmembrane glycoprotein gp41 are embedded into this lipid bilayer, together with cellular membrane proteins from the host cell, including Major Histocompatibility Complex (MHC) molecules, actin or ubiquitin [18]. A matrix shell comprising about 2,000 copies of p17 matrix protein (MA) is internally coating the viral envelope. The innermost structural layer is the capsid, which is composed by approximately 2,000 copies of p24 capsid protein (CA), and confers the icosahedric shape of the virus core. The capsid contains the enzymatic machinery required for the early steps of the viral replication, namely the Reverse Transcriptase and the Integrase, as well as two copies of HIV-1 genomic RNA that are stabilized by 2,000 p7 nuclocapsid proteins (NC). The viral proteins Nef, Vif and Vpr, but not Rev, Tat and Vpu, are also packaged inside the virion.[19].

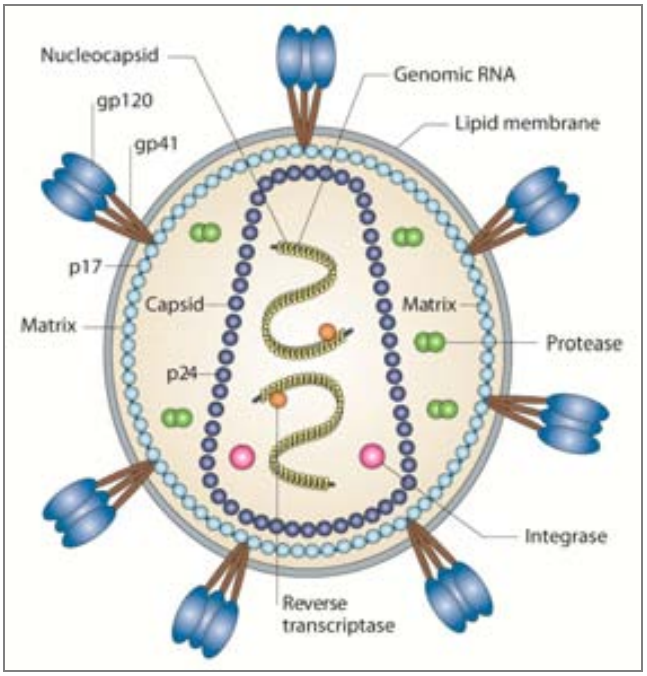


**Figure 2.** Schematic representation of the HIV-1 morphology showing the RNA genome and the viral proteins. *Adapted from Karlsson Hedstam, G.B., et al., 2008* [20].

### 1.5 Replication cycle of HIV-1

The HIV-1 replication cycle includes several stages, all of them potentially inhibited by antiretroviral drugs (Fig. 3).

- Virus entry into host cell: This stage requires the interaction of the HIV-1 gp120 with the CD4 receptor on the surface of the target cell [21]. Following CD4 binding, gp120/gp41 spike complex undergoes a structural change that enables the interaction of gp120 with a target chemokine receptor (mainly CXCR4 or CCR5), that acts as co-receptor. This allows for gp41 conformational change that leads to the virus-host cell fusion, and subsequent viral capsid internalization [22]. This fusion introduces the contents of the virion into the cytoplasm of the cell.
- Reverse transcription: Shortly after desencapsidation, the viral Reverse Transcriptase acts in the cellular cytoplasm and converts the ssRNA genome found in the virion into linear double-stranded DNA (dsDNA). During this process, the viral RNA template is also degraded by the Reverse Transcriptase RNase H activity, with the assistance of other viral and cellular factors. The newly synthesized proviral DNA is the substrate for the integration process [23].
- Nuclear import and integration: After the synthesis of viral DNA in the cytoplasm, it associates with viral Integrase and other proteins. This high-molecular-weight product, called preintegration complex (PIC), is later transported to the nucleus for subsequent integration. Then, the viral Integrase mediates the covalent attachment of the viral DNA into a host cell chromosome. Once integrated, the proviral DNA is replicated along with cellular DNA during cycles of cell division, as with any cellular gene [24]. However, depending on the activation status of target cells, the integrated viral DNA can remain latent, in a state of reversibly non-productive infection, or it can undergo active virus production.
- Transcription and translation: The integrated HIV-1 provirus acts as a transcription template. Immediately after infection, the cellular machinery produces only short completely spliced messenger RNA (mRNA) encoding the viral proteins Tat and Rev. The mRNA transcripts are transported out of the nucleus for translation. Control of HIV-1 gene expression depends on these two viral regulatory proteins. Then, transcription increases, and incompletely spliced mRNA of *env*, *vif*, *vpr*, and *vpu* genes are produced. The full-length unspliced mRNA for the Gag-Pol polyprotein and the RNA genetic material for new virons

are also synthesized. After that, the unspliced viral transcripts are also exported to the cytoplasm and translated [17].

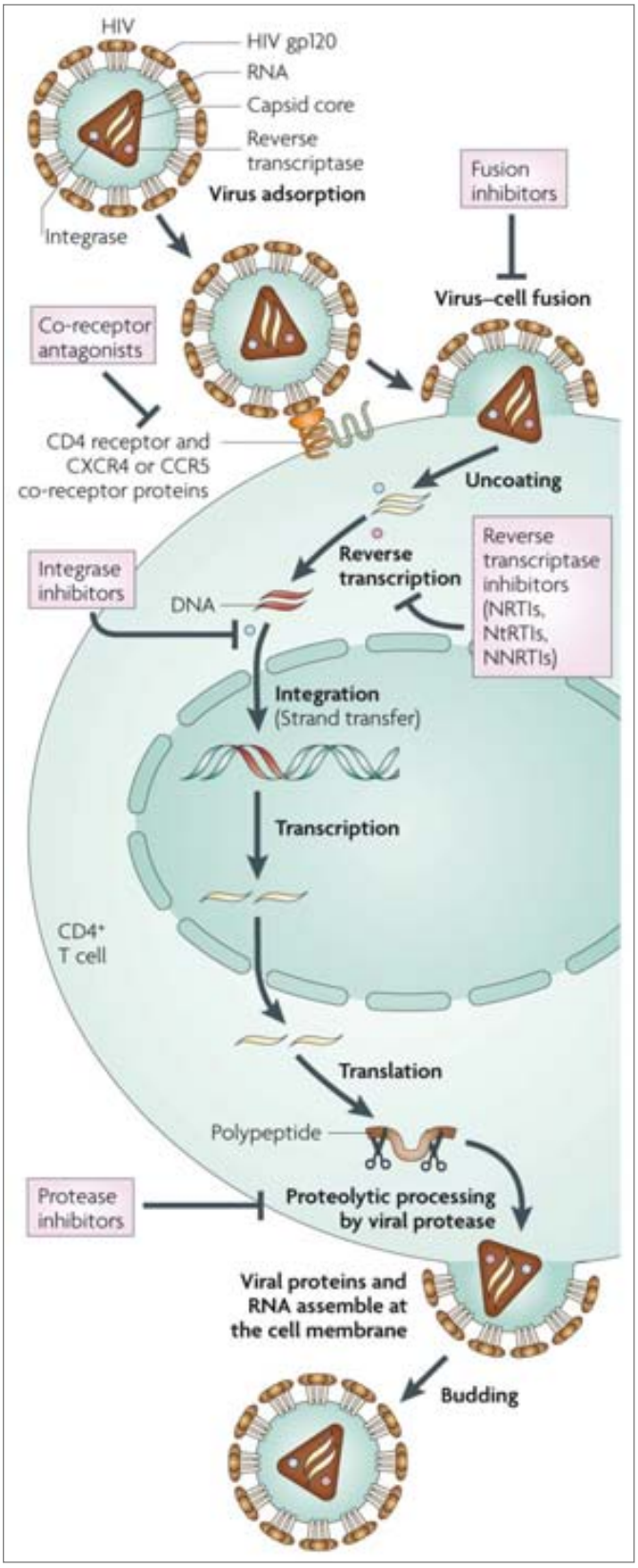


**Figure 3.** Schematic representation of the HIV-1 replication cycle and antiretroviral drug targets. There are different classes of antiretroviral drugs that act on different stages of the HIV-1 life cycle. Inhibitors that interact with either fusion or co-receptor binding inhibit the viral entry to the host cell. Reverse transcriptase inhibitors include nucleoside reverse transcriptase inhibitors (NRTI), nucleotide reverse transcriptase inhibitors (NtRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). Then, the crucial step of integration of the viral dsDNA into the host cell genome can also be blocked by integrase inhibitors. Finally, the protease inhibitors act in the cleavage of precursor polyproteins, hampering the proteolytic processing and the subsequent maturation of HIV-1 virions. *Reproduced from De Clerq, E., 2007* [26].

- Viral assembly, budding and maturation: Virion assembly occurs at specialized plasma membrane microdomains, and is mediated by the HIV-1 Gag polyprotein. This phase includes the plasma membrane binding, the protein–protein interactions to create spherical particles, the viral Env protein concentration, and the genomic packaging. On the contrary, the budding event, that releases the virion from the plasma membrane, is controlled by the host endosomal sorting complexes required for transport (ESCRT) machinery. Initially, the Gag polyprotein assembles into spherical immature particles and, as the immature virion buds, viral protease cleaves Gag into structural p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC), and p6 proteins, and SP1 and SP2 peptides. This process is required for conversion of the immature virion into mature infectious HIV-1 [25].

### 1.6 Course of HIV-1 infection *in vivo*

HIV-1 infects cells mainly of the immune system, preferentially $CD4^+$ T lymphocytes, but also monocytes, macrophages, dendritic cells and $CD8^+$ T cells. HIV-1 infection is characterized by gradual decline in $CD4^+$ T cells, chronic immune activation and subsequent loss of immunological competence, eventually culminating in AIDS. The natural course of HIV-1 infection usually takes a period of 8 to 10 years before clinical manifestations of AIDS occur. However, there is high variability among patients, and sometimes this period may be 2 years or less. Three different phases can be distinguished during the HIV-1 course of infection in the absence of antiretroviral therapy (Fig. 4):

1. Acute infection: This stage lasts from 2 to 6 weeks after infection, until anti-HIV-1 antibodies are detectable (seroconversion). During this phase, HIV-1 rapidly replicates resulting in a marked increase of viral load ($10^5$-$10^7$ HIV-1 RNA copies/ml), while a massive depletion of $CD4^+$ T lymphocytes occurs. The majority of recently HIV-1–infected people experience some mononucleosis-like symptoms, such as fever, lymphadenopathy, myalgia, rash, pharyngitis, etc. However, about 30% remain asymptomatic during initial period of infection [27].
2. Chronic phase: Following the initial acute infection, the titers of viral load in blood decrease around 100 times, reaching a set point that is strongly predictive of the time of clinical disease onset. This second phase is characterized by continuous stimulation of the immune system by repeated exposure to viral antigens and altered cytokine environment. Despite the induction of innate and adaptive

immune responses, the virus continues replicating and disseminates into the lymphoid organs. The HIV-1–infected patient can remain in a clinical asymptomatic latency for several years. In general, after 10 years of infection in the absence of antiretroviral treatment, the 50% of HIV-1–infected individuals develop signs of infection, including a marked decrease in $CD4^+$ T cells, lymphoid hyperplasia and impairment of immune functions [28].

3. AIDS: The progressive immune deficiency culminates in the development of AIDS. In this stage, the $CD4^+$ T cell counts drops below 200 cells/µl, viral load rapidly rises and immune responses sharply fail. Hence, this scenario enables the onset of opportunistic infections and HIV-1–associated cancers, such as Kaposi's sarcoma or Burkitt's lymphoma, which finally results in death.

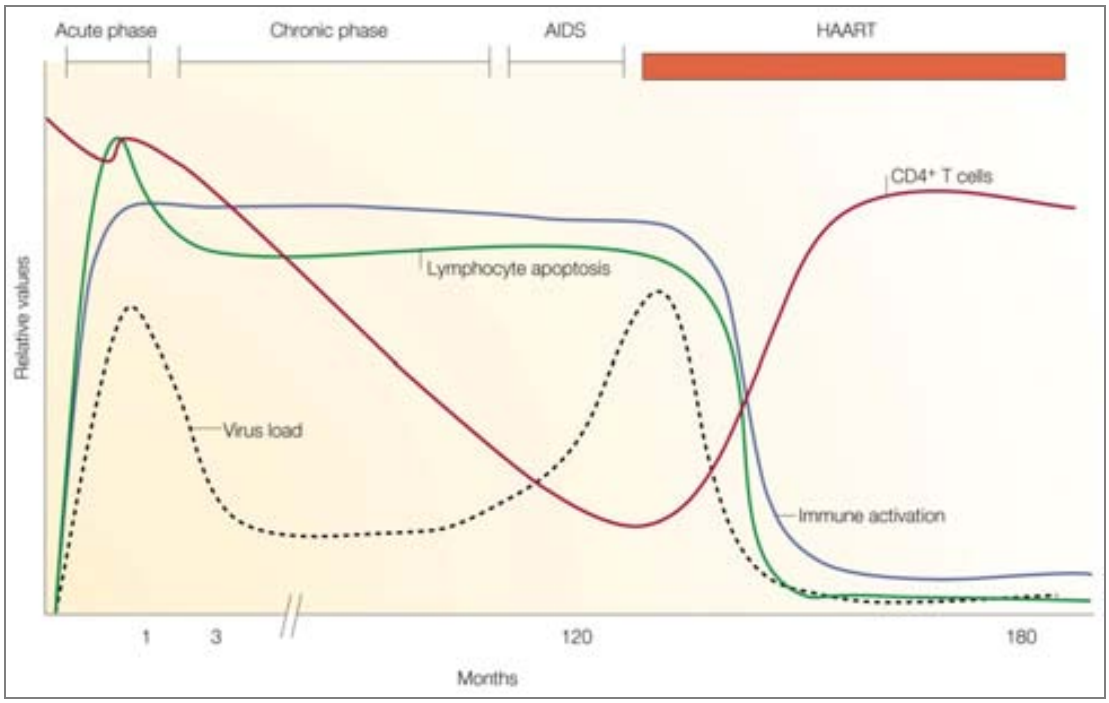


**Figure 4.** Schematic representation of the natural HIV-1 course of infection, showing the relationship between viral load, virus-mediated immune activation, T-cell apoptosis and disease progression before and after anti-retroviral therapy. *Reproduced from Gougeon, M.L., 2003* [28].

Nowadays, there is not a cure or effective vaccine against AIDS. Nevertheless, the highly active antiretroviral therapy (HAART) has modified the natural history of HIV-1 infection. On HAART, the virus replication is strongly suppressed and the $CD4^+$ T cell counts rise. Moreover, the antiviral treatment is also associated with a downregulation of immune activation, a reduction in the incidence of opportunistic infections and HIV-1–related tumors and a decrease in AIDS morbidity and mortality [28].

## 2. Dendritic cells

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) in the immune system and act as a link between innate and adaptive immunity [29, 30]. DC reside as immature cells in peripheral tissues, where they monitor their environment for danger signals, capture pathogens, and migrate to draining secondary lymph nodes (Fig. 5 A). During migration, DC process captured antigens while acquiring a mature phenotype by upregulating co-stimulatory and major histocompatibility complex (MHC) molecules at the cell membrane. Once they reach the T-cell areas of the lymph nodes, mature DC (mDC) can present pathogen-derived peptides to naïve T cells in association with HLA molecules. This process initiates an adaptive cellular immune response that involves $CD4^+$ T helper cells ($T_H$) and cytolytic $CD8^+$ T lymphocytes (CTL) and a humoral immune response that requires activation of naïve [31] and memory [32] B cells. During this process, the $CD4^+$ $CD25^-$ regulatory T cell populations also proliferate to control the immune response [29, 33]. In addition, DC can contact with other innate immune cells, such as natural killer (NK) cells [34], natural killer T (NKT) cells [35] and γδ T cells [36]. Such interactions may modulate the quality and strength of the subsequent adaptive response [37, 38].

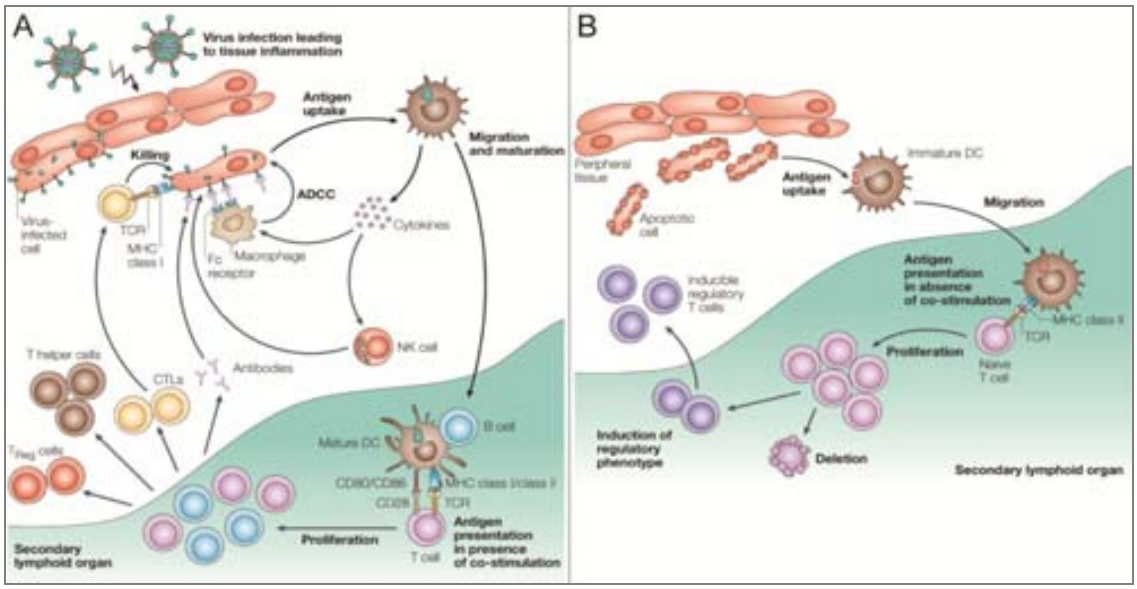


**Figure 5.** Mechanisms of action of immature and mature DC in vivo: (A) mDC induce immunity, while (B) immature DC mediate tolerance. *Adapted from Banchereau, J. and Palucka A.K., 2005* [33].

Besides the function of DC in the establishment of adaptive immune responses, they are also essential mediators in the induction and maintenance of immune tolerance (Fig. 5 B). While central tolerance depends on mature thymic DC during T-cell development, peripheral tolerance is mediated by immature DC (iDC) in lymphoid organs [33]. To that end, iDC present tissue antigens in the absence of appropriate co-stimulation, leading to either the T-cell anergy or deletion [39] or the generation of interleukin-10-inducible regulatory T cells [40, 41].

### 2.1 DC subsets

DC constitute a heterogeneous population of bone-marrow-derived cells that include the $CD11c^+$ $CD123^-$ myeloid DC (myDC) and the $CD11c^-$ $CD123^+$ plasmacytoid DC (pDC) [30]. Although all DC share many common features, they represent phenotypically different subtypes, with distinct life-span, anatomic location, and function. The myDC are present in blood, and in lymphoid and nonlymphoid tissues. According to their tissue localization, myDC are divided into skin DC including Langerhan's cells (LC) in epidermis and interstitial DC in dermal areas; mucosal tissue-associated DC; lymphoid tissue-associated DC including splenic marginal zone DC, T-cell zone-associated interdigitating cells, germinal center DC, and thymic DC; and interstitial tissue DC including liver DC and lung DC [42]. The pDC are located in blood, lymph nodes, mucosal-associated lymphoid tissue and spleen and are capable of secreting large amounts of type I interferon (IFN) in response to viral stimulation [43].

DC are present at very low frequencies in blood, constituting 0,5-2% of total peripheral blood mononuclear cells (PBMC) [44]. In addition, skin DC cannot be isolated in an immature state since they mature as a result of extraction [45]. Interestingly, monocytes can originate DC *in vivo* after sensing inflammatory signals, and contribute to the replenishment of DC in the host [46]. Consequently, the most common model used not only for studying the immunology of myDC but also for clinical DC vaccination [47] is monocyte-derived dendritic cells (MDDC) [48, 49]. $CD14^+$ monocytes cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) differentiate into immature MDDC, with features similar to myDC [48, 49]. However, immature MDDC seem to display higher expression of MHC and costimulatory molecules compared to immature myDC. Then, immature MDDC can turn into mature MDDC after exposure to various activating stimuli, including pathogen-related molecules (e. g. lypopolysaccharide (LPS), dsRNA, polyinoisic:polycytidylic acid (polyI:C)), inflammatory cytokines (e. g. tumor necrosis

factor α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interlukin-10 (IL-10), transforming growth factor β (TGF-β), and prostaglandins) or T-cell-derived signals (e. g. CD40L) [50-53].

### 2.2 DC in innate immunity: initiation of an immune response

The innate immune system is the first line of defense against pathogens or tumoral cells. It confers immediate, short-lived and non-specific immunity on the organism, protecting it from disease. It consists of cellular and biochemical factors that are in place even before infection, ready to response rapidly to invading pathogens or injured cells. The innate immune responses against infections are elicited by constitutive and conserved structures that are common to groups of related microbes, called pathogen-associated molecular patterns (PAMP). PAMP are produced only by microbes, and not by host cells, allowing the distinction between “self” and “non-self” by the innate immune system. Consequently, the detection of PAMP signals the presence of infection, because they are molecule signatures of microbial invaders. The innate immune system detects these danger signals through the pattern-recognition receptors (PRR), which can be expressed on the cell surface, in intracellular compartments, or secreted into the blood and tissue fluids [54].

As critical effectors of innate immunity, DC are situated at the portals of human body, such as skin, mucosal surfaces and the blood, so that they encounter invading pathogens early in the course of infection. Consequently, DC sample their environment for PAMP, and after PAMP recognition, PRR induce intracellular signaling that leads to DC maturation, cytokine production and migration towards lymph nodes. Once there, DC would link innate and adaptive immune systems, by triggering highly specific adaptive immune responses against the particular pathogen. Several PRR have been shown to participate in the recognition of pathogens by DC (Fig. 6): Toll-Like Receptors (TLR), C-type lectin receptors (CLR), nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), and retinoic acid-inducible gene I (RIG I)-like receptors (RLR). The expression pattern of PRR differs between DC subsets but also within the cell, given that some are expressed on the cell surface and others intracellularly. However, these PRR can function synergistically to induce and regulate the innate immune responses [55-57].

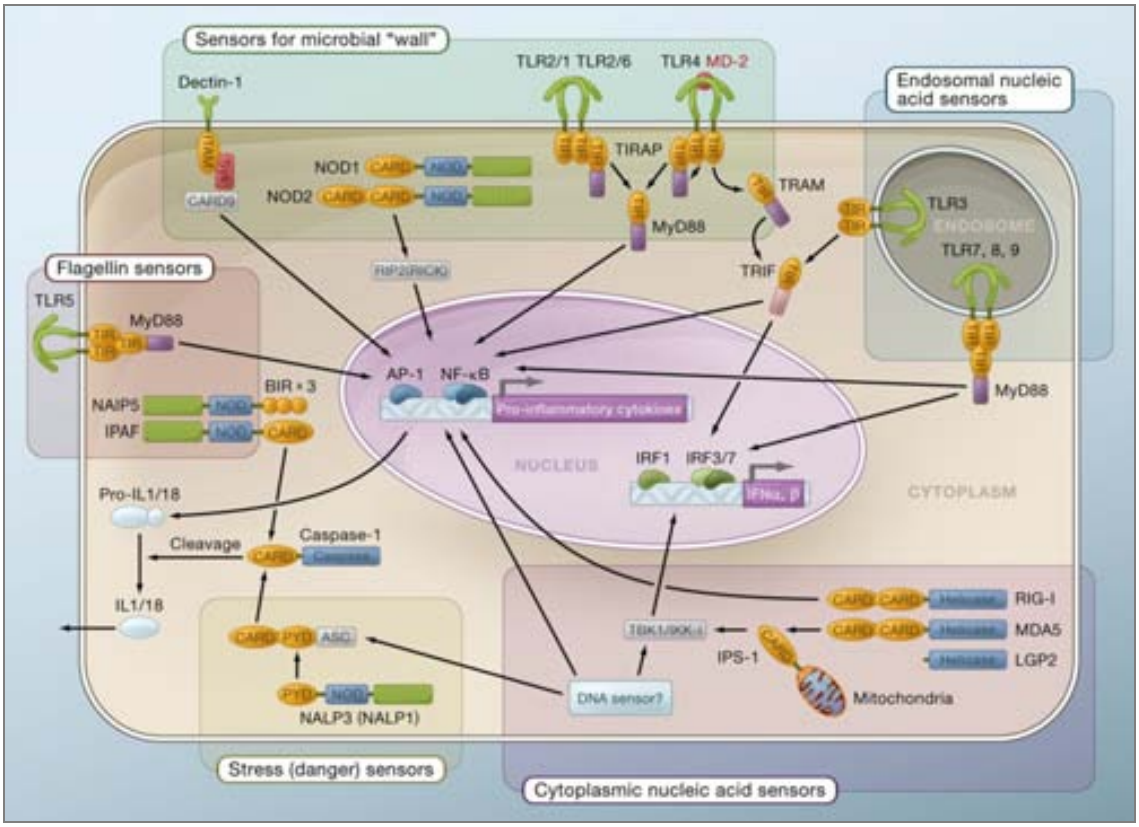


**Figure 6.** Sensing of PAMP by host innate immune receptors: Toll-Like Receptors (TLR), C-type lectin receptors (CLR), nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), and retinoic acid-inducible gene I (RIG I)-like receptors (RLR). They cooperate to recognize a variety of microbial infection by sensing microbial cell walls, bacterial motor flagellin, microbial and modified host nucleic acids, or stress-induced molecules on the cell surface, in the endosomes, or in the cytosol. *Reproduced from Ishi, K.J. et al., 2008* [58].

### *2.2.1 Toll-Like Receptors (TLR)*

The TLR comprise a family of type I transmembrane receptors, characterized by an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/Interleukin-1-receptor (TIR) domain [59]. Human TLR family consist of 10 members (TLR1-10) that recognize not only PAMP, including proteins, lipids and nucleic acids derived from viruses, bacteria, parasites, and fungi, but also damaged host cell components, such as nucleic acids [58]. TLR are found in the cellular membranes, either in the plasma membrane (TLR1, -2, -4, -5, -6 and -10), or in endoplasmic reticulum (TLR3, -7, -8 and -9) (Fig. 6). The differential intracellular location also involves diverse PAMP sensing. While the cell-surface TLR recognize bacterial and viral lipids and proteins, endosomal TLR sense nucleic acids. Some TLR form heterodimers, such as the TLR1/TLR2 and TLR2/TLR3 complexes, to recognize the lipoproteins and peptidoglycans (PGN) from Gram-positive bacteria. Conversely, TLR4 homodimerizes and requires the accessory myeloid differentiation protein-2 (MD-2) to detect the bacterial LPS and lipoteichoic acid

(LTA). Regarding the endosomal TLR, TLR3 recognizes dsRNA, TLR7 and TLR8 respond to ssRNA, and TLR9 is responsible for the recognition of viral and bacterial unmethylated CpG DNA [60, 61]. All TLR signal through the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway, except for TLR3, that uses the Toll/interleukin-1 receptor (TIR)-domain-containing adaptor-inducing interferon-β (IFN-β) (TRIF)-dependent pathway, and TLR4, that activates both. Nevertheless, both MyD88- and TRIF-dependent cascades induce the production of proinflammatory cytokines and type I interferons (IFN-α and β) and regulate the immune responses [58, 62, 63]. Besides, the diversity and regulation of TLR response against PAMP is also controlled by the different TLR expression profiles between DC subsets, suggesting division of functions. myDC express all 10 TLR with the exception of TLR9, whereas pDC complements the TLR repertoire with the expression of TLR9 together with TLR1, TLR6 and TLR7 [64-67].

### *2.2.2 C-type lectin receptors (CLR)*

The C-type lectin receptors (CLR) are a group of $Ca^{2+}$-dependent (C-type) carbohydrate-binding (lectin) proteins with highly conserved carbohydrate recognition domains (CRD) (Fig. 6). The CLR family comprises at least 17 subgroups, mainly with adhesion functions. However, type II, V, and IV are present in myeloid cells and act as PRR usually binding mannosylated antigens [68, 69]. These CLR not only serve as antigen receptors but also regulate the migration of DC and their interaction with T lymphocytes [70]. According to the orientation of the N-terminus of the protein, the CLR can be classified into two groups. On the one hand, type I CLR have their amino-terminus outwards from the cell membrane and contains several CRD or CRD-like domains. On the other hand, type II CLR has the N-terminal pointing into the cytoplasm and a single C-terminal CRD [70]. The most common type I CLR in DC are macrophage mannose receptor (MMR; CD206) and DEC-205 (CD205), which contain a tyrosine-based motif for internalization in clathrin-coated vesicles [71, 72]. On the contrary, several members of type II CLR are expressed by DC, some of them restricted to specific DC subsets, suggesting distinct functions. Langerin (CD207) is only expressed by LCs in the epidermis [73] while Dendritic-cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN; CD209) is present in dermal DC and DC in lymph nodes but absent in LC [74]. However, both recognize the monosaccharides mannose, fucose, N-acetyl-glucosamine (GlcNAc) and the olicosaccharide mannan [70, 75], which are highly abundant in pathogens such as HIV-1 [76], *Mycobacterium* species

[77], *Candida* species [78], *Helicobacter pylori* and helminth structures [79]. Other type II CLR also expressed by DC are DC immunoreceptor (DCIR), DC-associated C-type lectins 1 and 2 (dectin-1, dectin-2), C-type lectin receptor 1 (CLEC-1), lectin-like immunoreceptor (LLIR), and Blood DC antigen 2 (BDCA-2) [70]. CLR expression profile not only depends on DC subsets or tissue localization, but also on the maturation status of the cell. Thus, activation increases the expression of DEC-205 on blood DC, LC and MDDC, while MDDC decreases the production of MMR and DC-SIGN decreases on upon maturation [80].

### *2.2.3 Nucleotide-binding oligomerization domain (NOD)-like receptors (NLR)*

The Nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) are cytoplasmic soluble PRR that recognize intracellular PAMP and danger signals (Fig. 6). The NLR are normally present in the cytoplasm in an inactive, auto-repressed form. Upon direct or indirect binding of a PAMP, the receptor undergoes a conformational rearrangement that triggers oligomerization. The NLR family can be divided in five groups: NLRA, NLRB, NLRC3–5 (including NOD1 and NOD2), NLRP1-14, and NLRX [81]. NLR share some ligands with TLR, such as the bacterial wall components and flagellin. Moreover, NLR and TLR can interact at the intracellular signalling level, allowing for synergistic innate immune responses [58, 82]. NOD1 and NOD2, which are expressed by DC [82, 83], receptors are involved in the detection of bacterial molecules produced during the synthesis and/or degradation of peptidoglycan [84], important for the induction of innate immunity against *Streptococcus pneumonia* [85], *Mycobacteria tuberculosis* [86] and *Listeria monocytogenes* [87]. Upon ligand recognition, NOD1 and NOD2 lead to nuclear factor κB (NF-κB) activation, while the rest of NLR from a large intracellular complex, known as imflammasome, that triggers inflammatory caspase-1 [88, 89].

### *2.2.4 Retinoic acid-inducible gene I (RIG-I)-like receptors (RLR)*

The Retinoic acid-inducible gene I (RIG-I)-like receptors (RLR) constitute a family of three cytoplasmic RNA helicases (Fig. 6): RIG-I, melanoma differentiation associated gene 5 (MDA5), and LGP2 [90]. RIG-I and MDA-5 sense dsRNA either form dsRNA viruses or ssRNA viruses, in which dsRNA is a replication intermediate. Both RIG-I and MDA-5 are able to induce antiviral responses by triggering the production of

type-I interferons (IFN-α and β) [91]. On the contrary, LGP2 acts as a regulator of RIG-I and MDA-5-mediated antiviral responses [92, 93]. Interestingly, RLR are the major sensors for viral infection in myDC, while in pDC TLR play a more important role.

### 2.3 DC in adaptive immunity: induction of specific immune responses

DC are key effectors in bridging innate and adaptive immunity. After antigen capture and PRR signaling, iDC sense and process the information and undergo a series of phenotypic and functional changes for becoming competent APC [29]. This maturation process is characterized by morphology changes, acquisition of high cellular motility, chemokine and cytokine secretion, downregulation of endocytic and phagocytic receptors, upregulation of costimulatory molecules, and translocation of major histocompatibility complex (MHC) molecules from intracellular compartments to the cell surface [30, 94]. As a result, DC process captured antigens into small peptides while migrating from peripheral tissues towards draining lymph nodes [30]. Once there, DC are able to stimulate specific immune responses by presenting these processed peptides to naïve T cells, inducing cellular immune responses that involve $CD4^+$ $T_H$ cells and CTL. In addition, DC activate naïve [31] and memory [32] B cells, launching humoral immunity, and NK cells [34], NKT cells [35] and γδ T cells [36].

#### *2.3.1 Antigen processing and presentation by DC*

The central issue for optimal T-cell activation is the recognition of antigens in the peptide-binding groove of surface-expressed MHC class I and class II molecules by specific T-cell receptors [95]. MHC class I molecules present peptides to $CD8^+$ T cells. These peptides derive from intracellular proteins degraded in the cytosol by the proteasome and comprise pathogens replicating in the cytoplasm or endogenous proteins synthesized by the cell itself (Fig. 7) [96]. On the contrary, MHC class II molecules present antigen-derived peptides to $CD4^+$ T lymphocytes. The precursor proteins are exogenous material that is endocytosed from the extracellular environment, and also endogenous molecules such as plasma membrane proteins, components of the endocytic pathway and cytosolic proteins that access the endosomes by autophagy. In endosomal compartments, proteins are degraded by cathepsins and other hydrolytic enzymes (Fig. 7) [97, 98]. Interestingly, DC have the unique ability to present exogenous antigens via MHC class I molecules *in vivo* (Fig. 7) [99, 100]. This process, called *cross*-presentation, plays an important role in the

induction of tolerance and antiviral and antitumor immunity [101]. These exogenous antigens can be endocytosed either by pinocytosis, phagocytosis or receptor-mediated endocytosis [98]. However, several receptors associated with pathogen recognition, such as CLR (CD205, Langherin or DC-SIGN) [102-104] or TLR [105-107], can capture antigens as well. The signaling associated with the pathogen sensing by PRR influences the dynamics of the antigen acquisition, processing and presentation. Finally, the efficiency of DC to present peptide antigens on MHC class I and II molecules depends on their ability to incorporate capture antigens into the correct processing compartments, resulting in functional specializations among DC subsets.

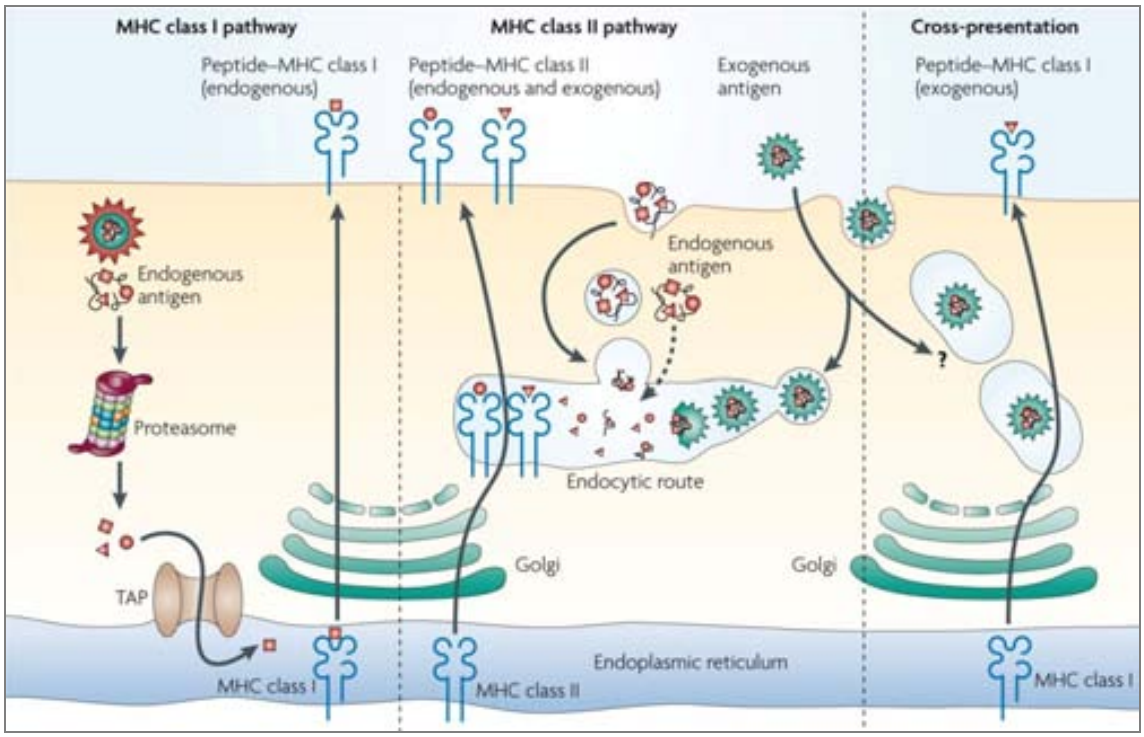


**Figure 7.** The antigen processing and presentation pathways in DC. *Left panel:* Classical endogenous MHC class I pathway, where the presented peptides derive from proteins degraded by proteasome in the cytosol. *Middle panel:* Classical exogenous MHC class II pathway, in which peptides are generated by proteolytic degradation in endosomal compartments. *Right panel: Cross*-presentation pathway in which exogenous antigens are delivered to MHC class I molecules, although the mechanisms involved in this pathway are still poorly understood. *TAP, transporter associated with antigen processing. Reproduced from Villadangos, J.A. and Schnorrer, P., 2007* [97].

#### *2.3.1.1 MHC class I presentation pathway*

The purpose of MHC class I presentation pathway is to report on intracellular events, such as infections or cellular transformation, to $CD8^+$ T cells. All nucleated cells express MHC class I molecules, which are composed of heavy chains and an invariant light chain, known as $\beta_2$-microglobulin. The antigen processing and presentation by MHC class I molecules can be divided into several stages [95]. It begins with the acquisition of antigens from proteins with errors, for example due to premature termination or misincorporation. Next, misfolded proteins are tagged with ubiquitin and degraded by proteolysis in the proteasome into peptides ranging between 8 and 16 aminoacids. The resulting peptides are then delivered to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) complex, and loaded onto newly synthesized MHC class I molecules. This process is supported by the peptide-loading complex, which is composed of a disulphide-linked dimer of tapasin and ERp57, calreticulin (CRT) and TAP molecules. Finally, peptide-loaded MHC class I molecules are exported to the cell surface via the Golgi complex and are presented to $CD8^+$ T lymphocytes [95]. The MHC class I pathway can be regulated by innate immunity, given that type I IFN produced after antiviral PRR signaling can induce the TAP complex, and, consequently, potentiate the antigen presentation to CTL [108].

#### *2.3.1.2 MHC class II presentation pathway*

The MHC class II pathway presents extracellular antigens to $CD4^+$ T cells. MHC class II molecules are dimmers of α- and β-chain that are assembled in the ER with the invariant chain (Ii), which enables proper folding and trafficking of the protein and protects the antigen binding-groove [109]. The exogenous antigens gain access to mildly acidic lysosomal MHC class II–rich compartments (MIIC) through phagocytosis and endocytosis, while endogenous proteins access the MIIC through autophagy. There, the MHC class II-Ii complexes accumulate [110]. The peptide antigens are generated following reduction of disulphide bonds by interferon-γ-inducible lysosomal thiol reductase (GILT) and cleavage by lysosomal proteases. In MIIC, HLA-DM promotes the sequential proteolysis of Ii and enhances the peptide binding to MHC class II molecules [111, 112] The degradation of Ii is regulated by the ratio between cathepsin S and its endogenous inhibitor cystatin C, which varies upon DC maturation, and allows the translocation of peptide-loaded MHC class II molecules to the plasma membrane [113]. However, the MHC class II transport and peptide loading in DC can

also occur in the absence of Ii [114]. In addition, several cytokines can regulate different stages of MHC class II pathway. For instance, endosomal proteolysis can be increased by IFN-γ, which upregulates cathepsin proteases, and IL-6 and IL-1β, that lower the endosomal pH [108]. On the contrary, IL-10 attenuates the proteolytic degradation by raising the pH of endosomes [115]. Moreover, the MHC class II molecules have a short half-life on the plasma membrane of iDC and are rapidly recycled. Conversely, DC maturation leads to a dramatic increase of peptide-MHC class II complex translocation to the cell surface, where can remain stable for days to be presented to $CD4^+$ T cells [94].

#### *2.3.1.3 Cross-presentation*

DC have the distinctive ability to *cross*-present exogenous antigens onto MHC class I molecules [99, 100]. To that end, DC peptide precursors need to access to the cytosol for processing by the proteasome, followed by their active transport into the ER where nascent MHC class I molecules are found [95]. Two main intracellular pathways for *cross*-presentation of exogenous antigens have been reported, and are referred to as the “cytosolic” and “vacuolar” routes [116]. While the cytosolic pathway seems to be sensitive to proteasome inhibitors [117], the vacuolar route is sensitive to inhibitors of lysosomal proteolysis [118, 119]. These suggest that antigen processing for *cross*-presentation can be performed either in proteasome or endocytic compartments. Nevertheless, the routes and mechanisms by which exogenous antigens can be presented through MHC class I molecules remain unclear. This process is highly relevant for the induction of tolerance and antitumor immunity and for the initiation of immune responses to viruses that do not infect DC [101].

### *2.3.2 The immunological synapse*

DC are pivotal in the generation of immunity and tolerance. Interactions between DC and T cells can occur in the presence or absence of cognate antigen, both leading to various T-cell responses [120-122]. The initial DC–$CD4^+$ T-cell contact is antigen-independent, given that DC–T cell adhesion precedes antigen recognition [120, 121]. In the absence of antigen, DC–$CD4^+$ T-cell interactions also lead to several T-cell responses, contributing to the homeostatic maintenance of naïve T cells [120, 122, 123]. The adaptive immune responses are initiated by the interaction of the appropriate peptide-major histocompatibility complex molecule complex (pMHC) on the DC with the

T-cell receptor (TcR), constituting the basis of the immunological synapse. The T-cell activation and function require this engagement with DC, or another APC, because the immunological synapse provides sustained T-cell signaling and activation that lead to T-cell priming and TcR downregulation. The immunological synapse consists of a highly stable and organized area of contact between the DC and the T cell, where pMHC-TcR as well as adhesion and co-stimulatory molecules play a major role [124, 125]. All the molecules involved in the immunological synapse are organized in distinct areas within the contact interface (Fig. 8). These areas are termed supra-molecular activation complexes (SMAC) [125]. The central region of the SMAC (cSMAC) is enriched in TCR-pMHC clusters and the downstream signaling effectors, such as protein kinase C-θ (PKC-θ), and co-stimulatory molecules CTLA4/CD28-CD80/CD86 [126]. The cSMAC is surrounded by a ring of lymphocyte function-associated antigen 1 (LFA-1) and its ligand intercellular adhesion molecule 1 (ICAM-1), defining the peripheral SMAC (pSMAC). Outside the pSMAC, the CD43 and CD45 concentrate into the distal SMAC (dSMAC) [127].

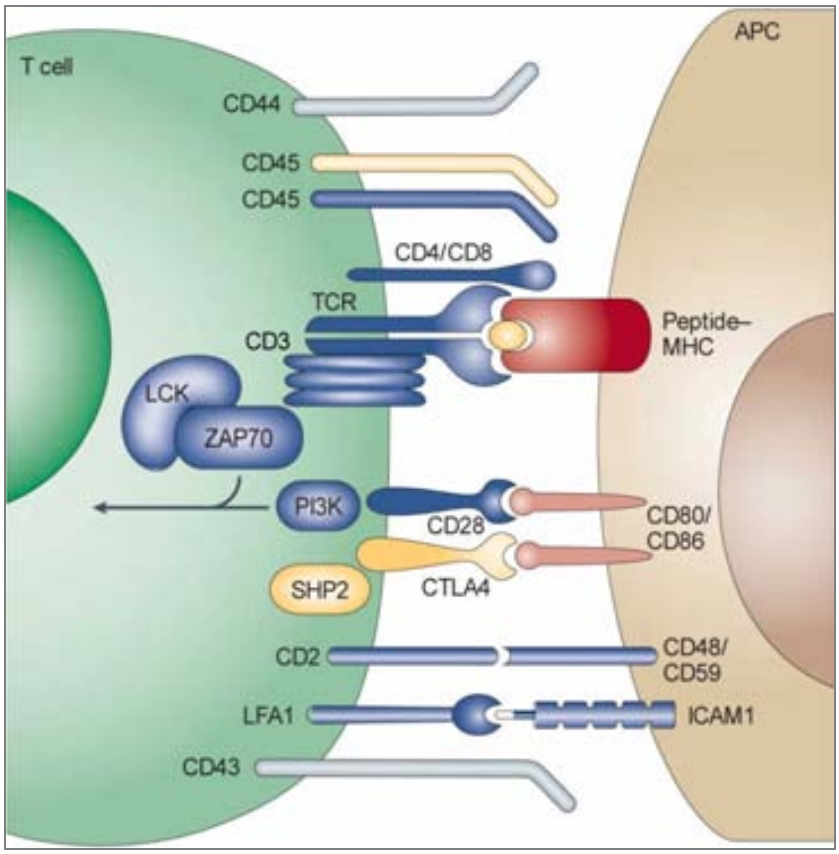


**Figure 8.** Schematic overview of a mature immunological synapse between an APC and a T cell, showing the key ligand pairs and singaling molecules involved in T-cell recognition. *Reproduced from Huppa, J.B. and Davis, M.M., 2003* [125].

DC maturation is decisive for the stability and duration of the initial contacts between DC and naïve T cells, and for the formation of the immunological synapse. While iDC establish multiple short low-affinity contacts with T cells, resulting in inefficient co-stimulation and calcium signaling, mDC form stable high-avidity conjugates with T cells, shaping the mature immunological synapse that effectively activates the T lymphocytes [128]. During DC–T cell contact, mDC receive feedback stimulation by naïve T cells that recognize the antigen they present [129]. Depending on the strength and pattern of stimulation and the cytokines present at the immunological synapse, $CD4^+$ T cells can differentiate into either T helper 1 ($T_H1$) and T helper 2 ($T_H2$) lymphocytes [130]. Consequently, the different classes of specific immune responses are driven by this biased development of pathogen-specific effector $CD4^+$ T-cell subsets that, in turn, activate different components of cellular and humoral immunity [131].

### *2.3.3 DC-mediated T-cell polarization*

DC not only alert the adaptive immunity to the presence of pathogens and danger singals, but are also able of tuning the adaptive immune responses (Fig. 9). The immunoregulatory role of DC mainly relies on the ligation of specific receptors that initiate and modulate DC maturation resulting in the development of functionally different effector DC subsets that selectively promote $T_H1$-, $T_H2$- or regulatory T-cell responses [131]. The $T_H$-cell stimulation and polarization requires three DC-derived signals. The first signal consists of TcR crosslinking with pathogen-derived pMHC complex on the cell surface of DC. This signal determines the antigen-specificity of the response, although the initiation of protective immunity requires the second co-stimulatory signal. This co-stimulatory signal two is largely mediated through the CD80 and CD86 molecules on DC, and determines the ability of naïve T cells to expand. In the absence of signal two, $T_H$ cells become anergic, which might lead to tolerance. DC can also select the type of immune response by expressing a selective set of T-cell polarizing molecules that determine the balance between $T_H1$-, $T_H2$- or regulatory T-cell development [131]. The nature of this third signal depends on tissue factors or activation of particular PRR in iDC, resulting in selective programming of these DC during maturation [132]. As a result, the adaptive immunity to pathogens is largely modulated by pathogen-derived compounds that functionally condition the DC for their expression of $T_H$-cell-polarizing molecules. In addition, the optimal and timely expression of the $T_H$-cell-polarizing molecules by DC requires feedback stimulation by

the CD40-CD40L interaction during intimate DC–T-cell contact [131]. The most documented $T_H1$-cell-polarizing factors involve the IL-12 family members, that include IL-12, IL-23 and IL-27 [133], the type I IFN-α and –β [134], and the cell-surface expressed intercellular adhesion molecule 1 (ICAM-1) [135]. On the other hand, $T_H2$-cell-polarizing molecules are the monocytic chemotactic protein 1 (MCP1 or CCL2) and the OX40 ligand (OX40L) [136], while the regulatory T-cell-polarizing factors comprises the IL-10 [137] and the transforming-growth factor-β (TGF-β) [131, 138].

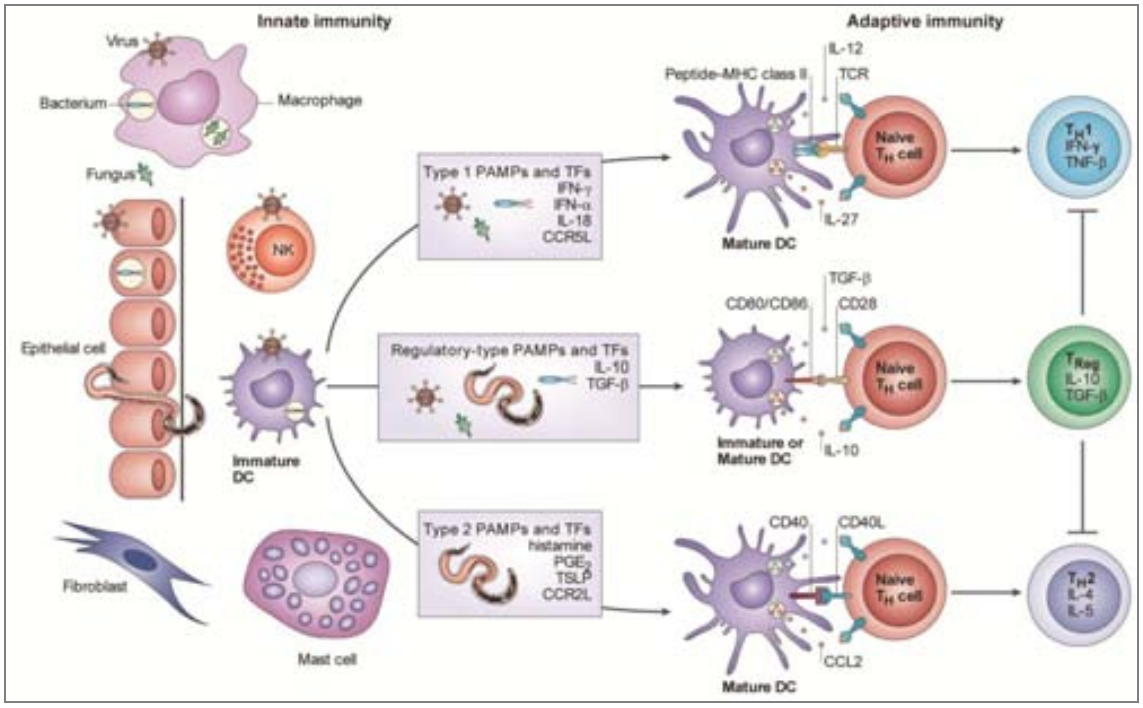


**Figure 9.** $T_H$ cell polarizing capacity of DC is influenced by the type of pathogen that is recognized. iDC can be polarized by type 1, type 2 and regulatory-type PAMP or tissue factors to become mature effector DC that promote the development of naïve T cells into $T_H1$, $T_H2$ or adaptive $T_{Reg}$ cells. Certain pathogens might inhibit DC maturation, resulting in regulatory iDC. DC-polarizing tissue factors can also be produced by various resident tissue cells and immune cells, including epithelial cells, NK cells, mast cells, macrophages and fibroblasts, depending on their origin or the way they are activated. *CCL:* CC-chemokine ligand; *CCR:* CC-chemokine receptor; *PGE2:* prostaglandin E2; *TcR:* T-cell receptor; *TF:* tissue factors; *TGF-β:* transforming growth factor-β; *TNF-β:* tumour-necrosis factor-β; *TSLP:* thymic stromal lymphopoietin. *Reproduced from Kapsenberg, M.L., 2003* [131].

## 3. Dendritic cell interactions with HIV-1

Sexual transmission through the lower genital and rectal mucosa accounts for the vast majority of new HIV-1 infections [12]. At portals of viral entry, such as the mucosa, DC are among the first cells to encounter HIV-1 when sexual transmission occurs [139, 140]. As potent APC, DC efficiently capture the incoming pathogens and move to lymphoid tissues, where they interact with $CD4^+$ T cells, the main targets of HIV-1 [140]. There, DC will induce protective adaptive immune responses, as well as tolerogenic responses [29]. However, HIV-1 has evolved ways to exploit DC, thereby facilitating viral dissemination and allowing evasion of antiviral immunity (Fig. 10) [140]. Consequently, DC, crucial in the generation and regulation of immune responses, play a dual role in HIV-1 infection by increasing the spread of HIV-1 while trying to trigger an adaptive response against viral infection.

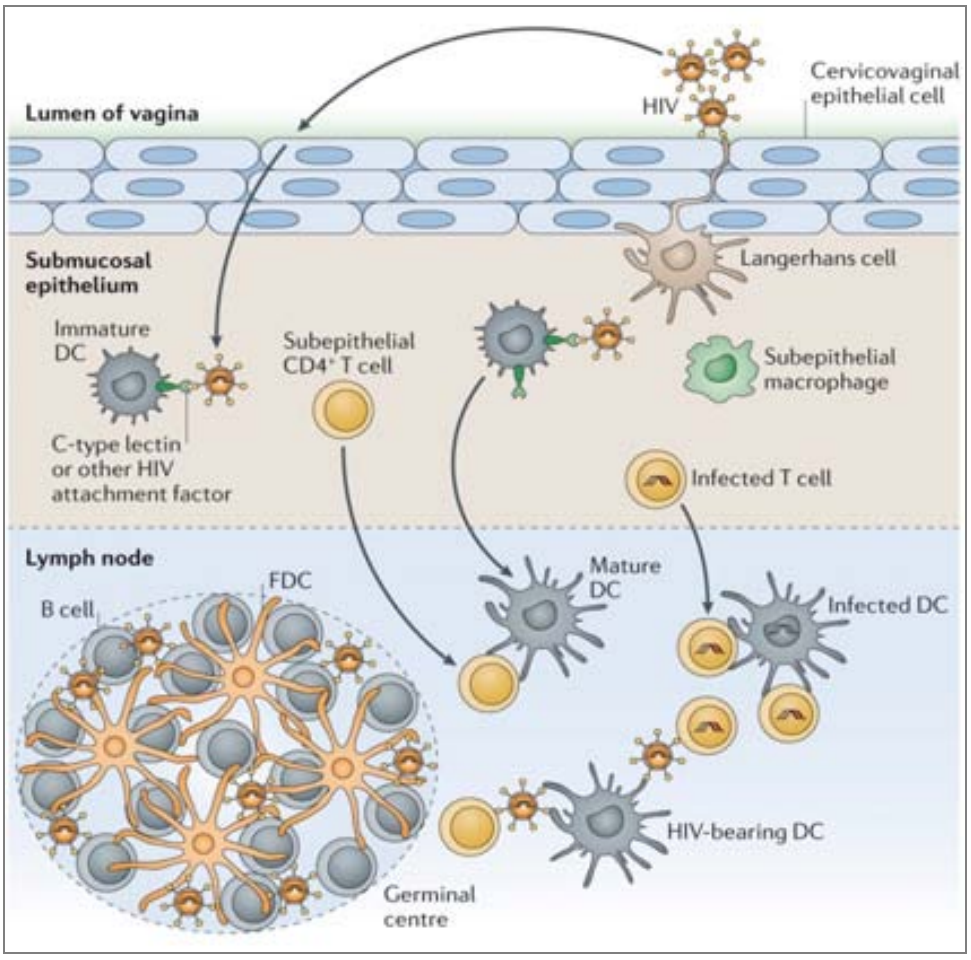


**Figure 10.** The role of DC in HIV-1 infection and dissemination. iDC capture HIV-1 at periphery, and then migrate to lymphoid tissues while acquiring a mature phenotype. Once there, mDC can facilitate HIV-1 dissemination by transmitting HIV-1 to $CD4^+$ T lymphocytes. *Reproduced from Wu, L. and KewalRamani, V.N., 2006* [140].

### 3.1 HIV-1 infection of DC

DC are susceptible to HIV-1 infection given that express the CD4 receptor and co-receptors [141-143]. Immature DC and certain mDC, for instance CD40L-matured DC, can be productively infected by R5-tropic viruses [144, 145]. However, HIV-1 replication in DC is less productive than in $CD4^+$ T cells, and the frequency of infected DC found *in vivo* is 10 to 100 times lower than $CD4^+$ T lymphocytes [146]. Furthermore, maturation of DC is associated with a decline in HIV-1 fusion [147], which, in turn, has a direct impact on the ability of mDC to support viral replication [144, 147], being 10 to 100-fold lower in mDC than in iDC [145, 148]. The inefficient HIV-1 productive infection of DC could be explained by the low levels of cell surface CD4 and co-receptors [141], the rapid and extensive degradation of internalized HIV-1 in intracellular compartments of DC [104, 149], and the expression of host factors that block HIV-1 replication, such as SAMHD1 [150, 151], BST-2/tetherin [152], and members of the APOBEC3 protein family [153-155]. Although all these factors hamper the HIV-1 replication in DC, thus controlling the viral dissemination, HIV-1 can subvert other mechanisms and pathways intrinsic to DC to gain access to target cells.

### 3.2 HIV-1 binding and capture by DC

The relative low expression of CD4 and co-receptors in DC [141] facilitates that, when HIV-1 encounters DC, virions interact with other DC receptors. Besides CD4 receptor, HIV-1 can bind to DC via gp120 through different attachment factors. Some CLR, such as DC-SIGN [76], Langerin [156], mannose receptor [156], and DCIR [157], mediate HIV-1 recognition by binding of high-mannose oligosaccharides on the heavily glycosilated viral Env [158-161]. DC-SIGN was first described as an efficient HIV-1 receptor that internalized HIV-1 particles to intracellular endosomal compartments, retaining their infectivity and facilitating the HIV-1 transmission from DC to T lymphocytes [76, 162-164]. However, DC-SIGN-mediated viral capture has been finally demonstrated to target HIV-1 to degradation pathways promoting MHC-I and MHC-II presentation of HIV-1–derived antigens [104, 165, 166]. In addition, syndecan-3, a DC-specific heparin sulfate proteoglycan (HSPG) [167], and the glycosphingolipid (GSL) galactosylceramide (GalCer) [168] act as receptors in the DC membrane by binding to the HIV-1 Env glycoprotein as well.

Interestingly, HIV-1 can also bind to DC in an Env glycoprotein-independent manner, by mechanisms yet unknown [169]. These Env-independent interactions seem

to be dependent on the GSL content of HIV-1 and/or DC [170-172], and are dramatically enhanced upon DC maturation with certain activation stimuli, such as LPS [144, 169, 172, 173]. As a result, LPS-matured DC (mDC LPS) (i.e., both monocyte-derived and blood-derived myeloid DC) capture greater amounts of HIV-1 compared to iDC [144, 169, 172, 173]. Furthermore, while DC maturation upregulates the Env-independent viral capture mechanism, the surface expression of DC-SIGN and other CLR diminishes [169-172]. Thus, mDC LPS are able to capture similar amounts of HIV-1 particles lacking Env than fully infectious Env-containing virions, and independently of DC-SIGN [169]. This Env-independent mechanism of HIV-1 capture has been suggested to contribute to the pool of infectious virus during the initial stages of HIV-1 infection.

As a result of this wide range of attachment receptors, which also differ between DC subsets and maturation status, the fate of incoming viral particles varies depending on the receptor to which HIV-1 engages to.

## 3.3 DC-mediated HIV-1 transmission

The classical model of HIV-1 spread involves binding of cell-free virions to permissive target cells. However, HIV-1 can subvert existing cellular communication pathways to enhance and potentiate viral propagation [174-177]. Direct cell-to-cell transmission provides advantages for retroviruses, by allowing them to obviate the dilution in the extracellular space that limits viral attachment [176, 178]. Cell-to-cell propagation could play a crucial role in the pathogenesis of HIV-1, since cell-mediated HIV-1 transmission is estimated to be 100 to 1,000 times more efficient than cell-free virus spread [179]. Indeed, mathematical models have predicted that cell-to-cell transmission of HIV-1 accounts for ~90% of new infection events in lymphoid tissue [180].

### *3.3.1* Cis-*infection vs* Trans-*infection*

DC can transmit HIV-1 to target cells by two major mechanisms: *cis*-infection and *trans*-infection [144, 149]. *Cis*-infection relies on the productive infection of DC, in which HIV-1 replicates and produces progeny virions that are released by DC to infect new target cells. During *trans*-infection, however, DC do not get infected but capture and internalize HIV-1 virions, preserving their infectivity, and finally transfer them to susceptible cells [76]. Cellular trafficking of HIV-1 might differ between iDC and mDC,

thus impacting on their ability to transmit HIV-1 to T lymphocytes. While iDC confine few HIV-1 particles in endosomes close to the plasma membrane, mDC accumulate whole virions in a perinuclear large vesicular compartment containing CD81 and CD63 tetraspanins but not the LAMP-1 lysosomic marker [163, 169, 172, 181]. Besides, HIV-1 transfer from DC to $CD4^+$ T cells displays a kinetic with two different phases [149]. During the first 24 hours after viral exposure, both iDC and mDC reroute the trapped virions to *trans*-infection, although it is temporally limited by viral degradation. Afterwards, the second phase depends on the productive infection of iDC, thus infecting target cells with HIV-1 synthesized *de novo* (*cis*-infection) [149].

*Trans*-infection is highly associated to DC given that HIV-1 replication in DC is inefficient, namely by several restriction factors. It has been proposed that HIV-1 takes advantage of a pre-existing exosome antigen-dissemination pathway intrinsic to mDC to enable final *trans*-infection of $CD4^+$ T cells [172], whereas others investigators adduce that only DC surface-bound HIV-1 are able to *trans*-infect target cells [182]. However, both models have been reconciled, demonstrating that mDC LPS concentrate HIV-1 in a tetraspanin-rich compartment that remains physically connected to the extracellular milieu [183]. By redirecting the intracellular trafficking, HIV-1 is able to escape from degradation and to be transmitted to $CD4^+$ T lymphocytes.

Generally, all maturation signals upregulate expression of HLA and costimulatory molecules, although the functional ability of the resulting mDC varies [184]. Consequently, depending on their qualitative maturation state, DC are able to polarize various T-cell responses [185]. The ability of mDC to transfer HIV-1 is also largely influenced by the maturation stimulus and the resulting DC subsets [186]. For instance, DC matured in the presence of prostaglandin E2 (PGE2) are inefficient in HIV-1 transmission to T lymphocytes [186], whereas other maturation factors, such as TNF, polyI:C and LPS, are very potent in transmission of HIV-1 [144, 186]. Indeed, the enhanced ability of mDC LPS to capture HIV-1 correlates with a greater capacity to transfer HIV-1 to susceptible target cells [144, 169, 173, 183, 186]. Accordingly, the study of DC activation with LPS in the context of HIV-1 infection is of great importance since plasma LPS levels are significantly augmented in chronically HIV-1–infected individuals as the result of increased microbial translocation [187]. Moreover, co-infections with gram-negative bacteria during HIV-1 infection may also enhance the LPS maturation of DC thus supporting the contribution of mDC to the spread of HIV-1 *in vivo* [188, 189].

### *3.3.2 Virological synapse vs Infectious synapse*

Productive HIV-1 infection results in viral transmission in *cis* via the formation of virological synapses [176, 190, 191], while HIV-1 *trans*-infection occurs through infectious synapses [149, 177]. Although the virological synapse has been extensively studied in the context of T cell–T cell viral transmission [176, 191, 192], infected iDC or macrophages can act alternatively as effector cells [193-196], while uninfected DC can also act as target cells [196, 197]. Cell adhesion in virological synapses is driven by the engagement of the CD4 molecule on the target cell with the viral Env on the surface of HIV-1–infected donor cells, which increases conjugate formation and favors viral transfer [176, 191, 192, 197]. Other authors have also suggested a role for leukocyte function–associated antigen (LFA-1), intercellular adhesion molecule 1 (ICAM-1), and intercellular adhesion molecule 3 (ICAM-3) in this kind of cell contacts [198].

On the contrary, the infectious synapse does not rely on productive infection of DC, but allows for viral transmission to target $CD4^+$ T cells through *trans*-infection [76]. After interaction with $CD4^+$ T cells, DC release the virions on the contact zone, which enables infection of the target cell [145, 169, 177, 183]. Although the involvement of DC-SIGN [76, 162], and LFA-1 [186, 199-201] is well documented, the cell-surface molecules that contribute to the formation of the infectious synapses have not been fully identified. Upon contact between DC and T cells, the CD4, CCR5, and CXCR4 receptors on the T cell and the HIV-1 harbored by the DC are recruited to the contact interface, thus facilitating HIV-1 transmission [177].

## 3.4 Impairment of DC-mediated antiviral response by HIV-1 infection

During viral infections, myDC generally produce IFN-α, IL-12 and IL-15 following the uptake of viral antigens (Fig. 11). These cytokines stimulate the production of IFN-γ by NK cells and promote the T-cell differentiation and survival. Depending on the cytokine signal, $CD4^+$ T cells differentiate into $T_H1$ or $T_H2$ cells. $T_H1$ cell-mediated IFN-γ secretion coupled with the IFN-γ produced by NK stimulate the activation of CTL and the production of immunoglobulin G2a by B cells. Conversely, $T_H2$ cell-mediated cytokine production stimulates immunoglobulin G1 antibody production by B cells but also inhibits activation of $T_H1$ cells. CTL along with NK cells contribute to the viral clearance inhibiting viral replication through the secretion of IFN-γ and killing infected cells releasing cytolytic mediators, such as granzymes or perforin. Similarly, virus-specific antibodies produced by B cells can inhibit viral replication by neutralizing and

preventing reinfection. Finally, pDC produce a first strong wave of IFN-α in response to viral infection, which potently enhances the NK cell-mediated cytotoxicity [202].

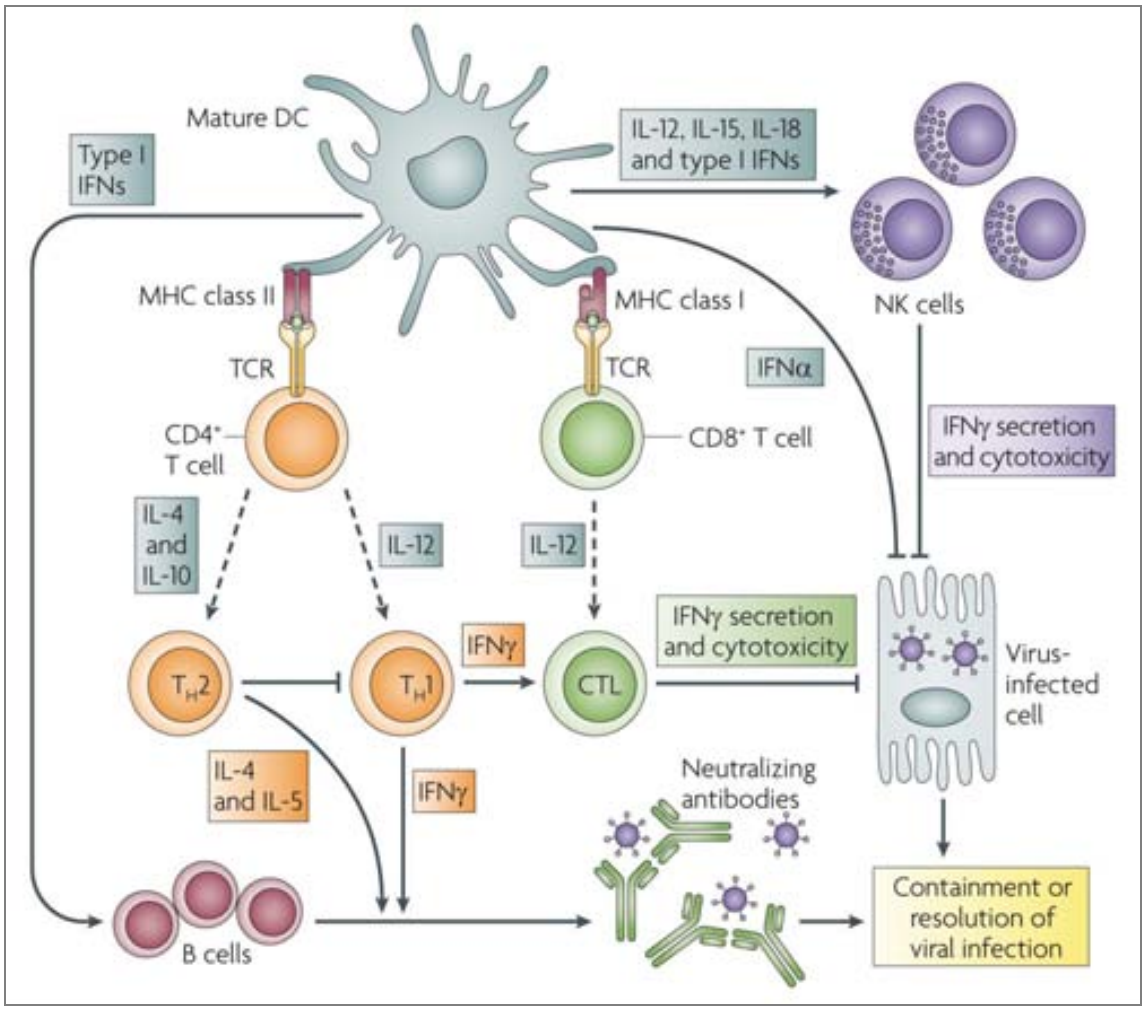


**Figure 11.** Function of DC in the immune response to viruses. *Reproduced from Lambotin, M., et al., 2010* [202].

Nevertheless, DC-mediated antiviral immune functions are altered during HIV-1 infection. Although HIV-1 replication is relatively inefficient in DC, viral engagement with different DC receptors alters the cellular function and induces immune dysregulation [203]. Maturation of DC is usually initiated upon recognition of a danger signal or incoming pathogen, upregulating migration and chemotaxis. However, HIV-1 is not able to fully mature DC [204, 205], with the exception of very high doses of virus [45]. This inability to mature DC probably dampens DC migration to lymphoid tissues and elicitation of DC-mediated cellular and humoral immune responses during HIV-1 infection [203], although the attachment of HIV-1 to CCR5 co-receptor has been shown

to increase chemotaxis and migration properties of DC [206]. In addition, autophagy, a central mechanism in the immunological control of intracellular pathogens, is inhibited in DC upon HIV-1 capture. The interaction of HIV-1 Env with DC induces a signaling cascade activating mammalian Target of Rapamycin (mTOR), which blocks autophagy, impairing the TLR-mediated innate immune responses and affecting antigen processing and MHC-II-mediated antigen presentation [207]. HIV-1 can also alter innate immune functions of DC by disrupting the interferon signaling pathways. In particular, HIV-1 infection significantly change gene expression in DC [208], leading to an increase in IRF-1 expression, required for viral replication in DC, and a failure in IRF-3 activation, which is impedes IFN production [209]. Furthermore, HIV-1 favours DC-mediated $T_H2$ immune responses against $T_H1$ responses, since the binding of viral envelope glycoprotein gp120 to the C-type lectin receptor DC-SIGN induces IL-10 production while reducing IL-12, IL-18 and IFN-γ secretion [207, 210, 211]. Finally, IFN-α production by pDC is altered resulting in a defective IFN-α-induced NK cell activity [203, 212].

# Chapter 2

# HYPOTHESIS & OBJECTIVES

Considering that maturation with LPS dramatically increases the ability of DC to capture HIV-1, which results in a high *trans*-infection to target cells, we hypothesize that the captured virions in mDC LPS could represent a source of viral antigens for HLA loading and T-cell activation. Therefore, elucidating the antigen presentation abilities of this DC subset, comparing with other maturation protocols and using a non-replicative virus, could provide new insights into DC biology and have implications in the optimization of DC-based immunotherapy against HIV-1 infection.

- **Objective 1:** To assess the efficiency of mDC LPS for presenting HIV-1–derived antigens to HIV-1–specific $CD4^+$ and $CD8^+$ T-cell clones, comparing with another maturation stimulus and evaluating the impact of the time lag between DC maturation and antigen loading on the stimulation of HIV-1–specific T-cell clones.
- **Objective 2:** To molecularly characterize the integrase-deficient HIV-1 isolate $HIV_{NL4\text{-}3\Delta IN}$ as an immunogen, analyzing the efficiency of DC for capturing and presenting $HIV_{NL4\text{-}3\Delta IN}$-derived antigens to HIV-1–specific $CD4^+$ and $CD8^+$ T-cell clones.

DC migrate from periphery to draining secondary lymphoid organs, which are enriched in $CD4^+$ T lymphocytes, the main target of HIV-1. There, DC induce and regulate adaptive immunity, and to do so DC continuously interact with other immune cells, such as $CD4^+$ T cells. Given that mDC LPS can efficiently mediate HIV-1 *trans*-infection, polarizing the HIV-1–containing compartment to the contact zone with $CD4^+$ T cells, and that the virological synapse between HIV-1–infected cells and uninfected cells is driven by the HIV-1 Env-CD4 interaction, we hypothesize whether the presence of HIV-1 or the antigen presentation would modulate the formation of mDC–$CD4^+$ T cell conjugates and the subsequent HIV-1 *trans*-infection.

- **Objective 3:** To explore the contribution of HIV-1 Env in the formation of conjugates between mDC and $CD4^+$ T cells at the infectious synapse and in the subsequent mDC-mediated HIV-1 *trans*-infection, analyzing its role in combination with adhesion molecules and antigen presentation.

The molecular determinants underlying the HIV-1 Env-independent uptake by mDC LPS remain uncharacterized. Consequently, clarifying the virus attachment factor and the DC receptor responsible for this mechanism could be crucial for the design of effective therapeutic strategies blocking the HIV-1 dissemination.

- **Objective 4:** To investigate the molecular determinants involved in the HIV-1 Env-independent binding and internalization mediated by mDC LPS.
- **Objective 5:** To identify the surface receptor on mDC LPS that enhances their Env-independent uptake of HIV-1 and their capacity to *trans*-infect HIV-1 to $CD4^+$ T cells.

# Chapter 3

# MATERIAL & METHODS

## 1. Primary cultures

### 1.1 Peripheral Blood Mononuclear Cells (PBMC)

Buffy coats from HIV-1–seronegative donors were purchased from the Banc de Sang i de Teixits (BST; Barcelona, Spain) or the Etablissement Français du Sang (EFS) of the Pitié-Salpêtrière Hospital (Paris, France). The institutional review boards of University Hospital Germans Trias i Pujol (Badalona, Spain) and Pitié-Salpêtrière Hospital (Paris, France) approved the studies. PBMC were obtained by a standard Ficoll-Hypaque (Lymphoprep, ATOM, AXIS-SHIELD PoC AS) density gradient centrifugation of heparin-treated venous blood (Fig. 12).

When autologous T lymphocytes were not needed for further experiments, blood was depleted of $CD8^+$ cells with RosetteSep Human CD8 Depletion Cocktail (StemCell) prior to the standard Ficoll-Hypaque density gradient centrifugation, in order to optimize monocyte recovery and isolation (Fig. 12 B).

### 1.2 Monocyte-derived Dendritic Cells

Monocyte populations were isolated from PBMC by positive selection using $CD14^+$ magnetic beads (Miltenyi Biotec), following the manufacturer's instructions, with a high purity (> 95% $CD14^+$) as determine by flow cytometry. Monocytes were cultured at a concentration of $8 \times 10^5$ cells/ml with Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco; Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco; Invitrogen), 1000 U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF), and 1000 U/ml interleukin-4 (IL-4) (both from R&D Systems) for 5 days to obtain iDC. Culture medium was replaced every two to three days by adding fresh GM-CSF, and IL-4. To obtain mDC, maturation stimuli were added to culture on day 5 and maintained until day 7: 100 ng/ml of lipopolysaccharide (LPS; SigmaAldrich), or 300 U/ml of interleukin-1 beta (IL-1β), 1000 U/ml of tumor necrosis factor alpha (TNF-α), 1000 U/ml of interleukin-6 (IL-6) (IL-1β, TNF-α and IL-6, all from CellGenix), and 1 µg/ml of prostaglandin E2 (PGE2; SigmaAldrich) (hereafter referred as ITIP), or Synthetic Monophosphoryl Lipid A VaccineGrade (MPLA; InvivoGen). mDC fully matured with LPS for 48 hours were designated as mDC LPS, and mDC fully matured with ITIP for 48 hours as mDC ITIP (Fig. 12 A). Cells were maintained at 37ºC in 5% $CO_2$ in a humidified incubator. All media contained 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen).

In experiments where autologous $CD4^+$ T cells were needed, remaining PBMC from negative fraction of monocyte isolation were cryopreserved in a liquid nitrogen tank with freezing medium consisting of heat-inactivated FBS containing 10% dimethyl sulfoxide (DMSO).

### 1.3 T lymphocytes

When needed (i.e. autologous and heterologous co-cultures), cryopreserved PBMC were thawn quickly but diluted slowly to remove DMSO from freezing medium. In order to avoid cell clumping, a treatment step with 50 U/ml Benzonase (Novagen) in RPMI-1640 containing 20% heat-inactivated FBS for 5 minutes at 37ºC was incorporated in the trawing protocol.

Untouched $CD4^+$ T cells were purified from fresh or frozen PBMC with an indirect magnetic labelling system (Miltenyi Biotec), according to manufacturer's instructions (Fig. 12 A). Purity of isolated populations (>95%) was assessed by flow cytometry. Cells were cultured at a concentration of $2 \times 10^6$ cells/ml in RPMI-1640 supplemented with 10% heat-inactivated FBS in the absence of any stimuli. When stated, $CD4^+$ T cells were stimulated for 72 hours in RPMI-1640 with 10% heat-inactivated FBS containing 10 U/ml interleukin-2 (IL-2; Roche Applied Science) and 3 µg/ml of phytohemagglutinin (PHA; SigmaAldrich). Cells were maintained at 37ºC in 5% $CO_2$ in a humidified incubator. All media contained 100 U/ml penicillin and 100 µg/ml streptomycin.

$CD4^+$ T cells used to generate HIV-1 derived from primary human T cells were obtained from 10 ml of buffy coat from three HIV-1–seronegative donors as described previously [213]. Briefly, blood was depleted of $CD8^+$ T cells using the RosetteSep Human CD8 Depletion Cocktail (StemCell) and, subsequently, enriched $CD4^+$ T cells were isolated by Ficoll-Hypaque density gradient centrifugation. Cells were then pooled and stimulated under three different conditions: 0.5 µg/ml low-dose PHA, 5 µg/mL high-dose PHA or plate-bound anti-CD3 mAb OKT3 (eBioscience) (Fig. 12 A). After 72 hours, cells were mixed together and used to generate $CD4^+$ T-cell derived HIV-1 viral stocks. To produce GSL-deficient HIV-1 viral stocks, enriched $CD4^+$ T cells remained unstimulated. Cells were maintained at 37ºC in 5% $CO_2$ in a humidified incubator. All media contained 100 U/ml penicillin and 100 µg/ml streptomycin.

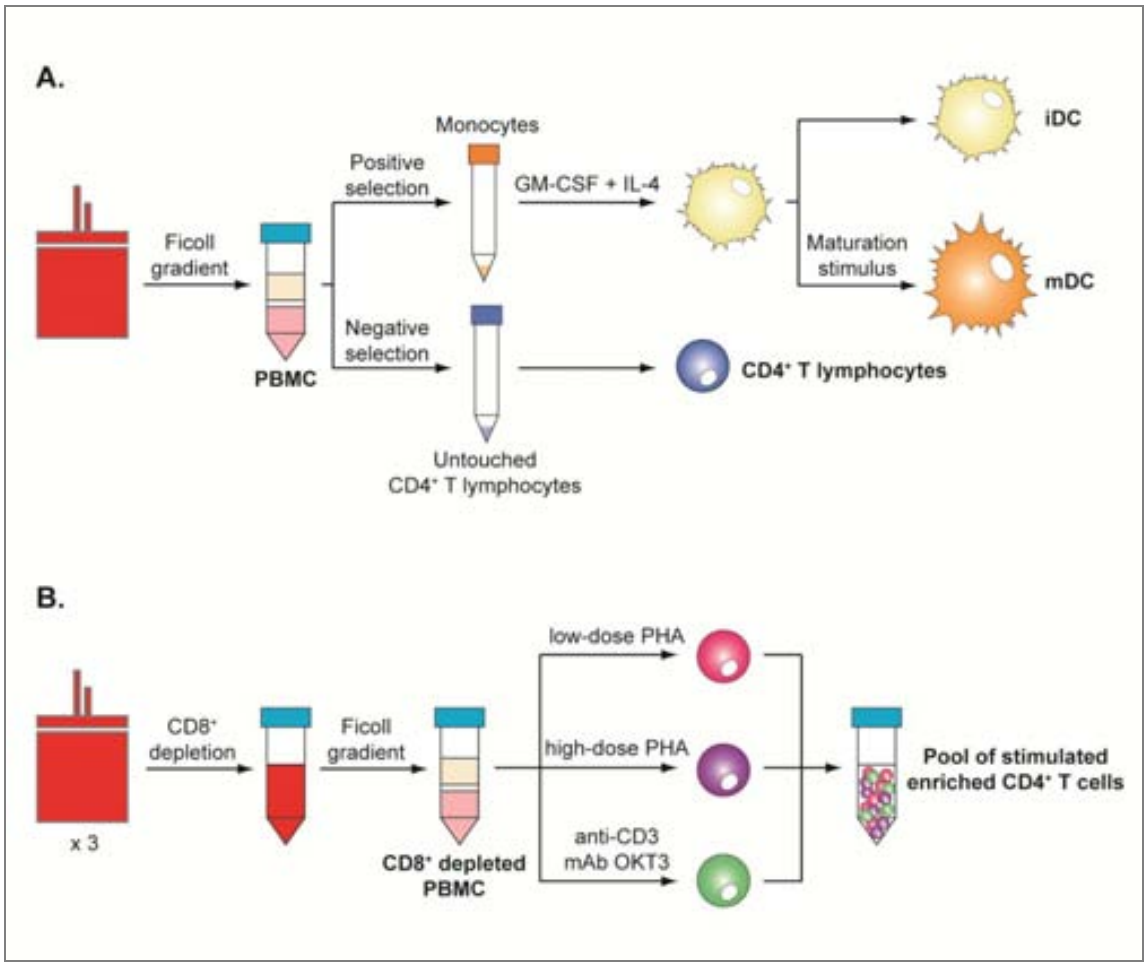


**Figure 12.** Schematic view of the general procedure followed to obtain **(A)** iDC, mDC and untouched $CD4^+$ T lymphocytes, or **(B)** pool of stimulated enriched $CD4^+$ T cells from PBMC of HIV-1 seronegative blood donors.

### 1.4 HIV-1–specific T-cell clones

The SL9-2 and EM40-F21 $CD8^+$ T-cell clones specific for HIV $p17^{Gag}$ (aa 77-85, SL9 peptide) and restricted by HLA-A*02 were used to evaluate HLA-I antigen presentation. The F12 and N2 $CD4^+$ T-cell clones specific for HIV $p24^{Gag}$ (aa 271-290) and restricted by HLA-DRβ*01/HLA-DRβ*04 and HLA-DRβ*04, respectively, were used to monitor HLA-II antigen presentation. The T-cell clones were restimulated and expanded as previously described [104, 165] using irradiated feeders and autologous lymphoblastoid cell lines loaded with cognate peptides in T-cell cloning medium (RPMI-1640 containing 5% serum AB (Institut Jacques Boy), 100 U/ml IL-2, and 1 µg/ml PHA (PAA) complemented with non-essential amino acid and sodium pyruvate (Gibco)). At least 4 hours before co-culture with DC, T-cell clones were thawed and allowed to rest in cloning medium without PHA. Cells were maintained at 37ºC in 5% $CO_2$ in a

humidified incubator. All media contained 100 U/ml penicillin and 100 µg/ml streptomycin.

## 2. Cell lines

### 2.1 Adherent cell lines

The TZM-bl (National Institutes of Health [NIH] AIDS Research and Reference Reagent Program: from J.C. Kappes and X.Wu, and from Tranzyme Inc.) [214-218], which contains separate integrated copies of the luciferase and β-galactosidase genes under control of the HIV-1 promoter, and HEK-293T (ATCC-LGC) [219-222] cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco; Invitrogen) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37ºC in 5% $CO_2$ in a humidified incubator.

### 2.2 Suspension cell lines

The T-lymphoblastoid MOLT-4/CCR5 (MOLT) cell line (NIH AIDS Research and Reference Reagent Program: from Dr. Masanori Baba, Dr. Hiroshi Miyake, Dr. Yuji Iizawa) [223], the derivative cell lines chronically infected with X4-tropic ($MOLT_{NL4-3}$) or R5-tropic ($MOLT_{BaL}$) HIV-1 [223-225] and the Jurkat (Clone E6-1) (NIH AIDS Research and Reference Reagent Program: from Dr. Arthur Weiss) [226] were cultured in RPMI-1640 with 10% heat-inactivated FBS and containing 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Cells were maintained at 37ºC in 5% $CO_2$ in a humidified incubator.

## 3. Immunophenotype

Monocytes were immunophenotyped after isolation from PBMC, while iDC and mDC LPS, mDC ITIP and mDC MPLA were stained at day 7. Before staining, cells were blocked with 1 mg/ml of human IgG (Baxter, Hyland Immuno) to prevent binding to Fc receptor through the Fc portion of the Ab. In order to evaluate the maturation

state of DC used in the antigen presentation assays, all DC conditions tested in the ELISPOT assays were immunophenotyped before launching the ELISPOT assay. Membrane molecules used for monocyte immunophenotyping were CD3, CD4, CD14, CD209 (DC-SIGN), and HLA-DR (Table 1); whereas the markers used for iDC and mDC immunophenotyping were CD4, C14, CD80, CD83, CD86, CD169, CD209 (DC-SIGN), HLA-ClassI or HLA-A,B,C, and HLA-DR (Table 1). Untouched $CD4^+$ T cells were immunophenotyped after isolation from PBMC by staining the CD3, CD4 and CD8 (Table 1) surface molecules. Comparative immunophenotype of monocytes, iDC and mDC LPS with surface markers CD14, CD4, DC-SIGN, HLA-DR, CD83 and CD86 is shown in Fig. 13.

The standard immunophenotype protocol consisted in cell staining with the corresponding fluorescently labelled mAb (Table 1) for 20 minutes at 4ºC to avoid Ab internalization, wash and resuspension in 2% formaldehyde solution. Matched isotype Ab controls were used. Samples were acquired on a FACSCalibur flow cytometer (BD Biosciences) and data analyzed using the FlowJo software (Tree Star).

**Table 1.** Monoclonal antibodies (mAb) used to immunophenotype monocytes, iDC, mDC and $CD4^+$ T lymphocytes by flow cytometry.

| Molecule name | Clone | Fluorochrome | Source |
|---|---|---|---|
| CD3 | SK7 | PerCP | BD Biosciences |
| CD4 | SK3 | FITC, PE and PerCP | BD Biosciences |
| CD8 | SK1 | PE | BD Biosciences |
| CD14 | M5E2 | FITC and PE | BD Pharmingen |
| CD80 | L307.4 | PE-Cy5 | BD Pharmingen |
| CD83 | HB15e | PE and APC | BD Pharmingen |
| CD86 | 2331 | FITC | BD Pharmingen |
| CD169 Siglec-1 (Sialoadhesin) | 7-239 | PE | AbDSerotec |

| | | | |
|---|---|---|---|
| CD209 (DC-SIGN) | DCN46 | PE and APC | BD Pharmingen |
| HLA-ClassI | W6/32 | FITC | SigmaAldrich |
| HLA-A,B,C | G46-2.6 | PE | BD Pharmingen |
| HLA-DR | L243 | PE | BD Biosciences |
| HLA-DR | G46-6 | PerCP | BD Biosciences |

## 4. Plasmids

The proviral construct pNL4-3 encoding for the replication-competent full-length CXCR4-tropic $HIV_{NL4-3}$ was obtained through the NIH AIDS Research and Reference Reagent Program from Dr. M. Martin [227]. The vector pNL4-3ΔIN, an HIV-1 Integrase-deficient vector derived from pNL4-3 lacking the whole Integrase coding region, was generated by Dr. M.J. Buzon [228] and was used to produce the non-replicative $HIV_{NL4-3\Delta IN}$. The pNFN-SX [229], a pNL4-3 construct expressing the CCR5-tropic HIV-1 JRFL envelope grycoprotein, was used to generate the replication competent full-length CCR5-tropic $HIV_{NFN-SX}$ and was kindly provided by Dr. W. A. O'Brien. The proviral construct pNL4-3.Luc.$R^-$.$E^-$ was obtained through the NIH AIDS Research and Reference Reagent Program from Dr. Nathaniel Landau [230, 231] to generate viral particles lacking the envelope glycoprotein $HIV_{NL4-3\Delta ENV}$. The pNL4-3Ren [232], containing the Renilla luciferase reporter gene, was a gift from Dr. S. Sánchez-Palomino to generate the infectious CXCR4-tropic reporter virus $HIV_{NL4-3Ren}$. The pCHIV and pCHIV mCherry [233], which were kindly provided by Dr. B. Glass and Dr. H-G. Kräusslich, were used to produce the $HIV_{NL4-3-Cherry}$.

The pCMV–BlaM-Vpr (Addgene plasmid 21950) [234], codifying for the HIV-1 Vpr protein fused to β-lactamase (BlaM), and the pAdVAntage vector (Promega), were used with pNL4-3 and pNL4-3ΔIN to generate HIV-1 virions containing the β-lactamase (BlaM)-Vpr chimera for viral fusion assays.

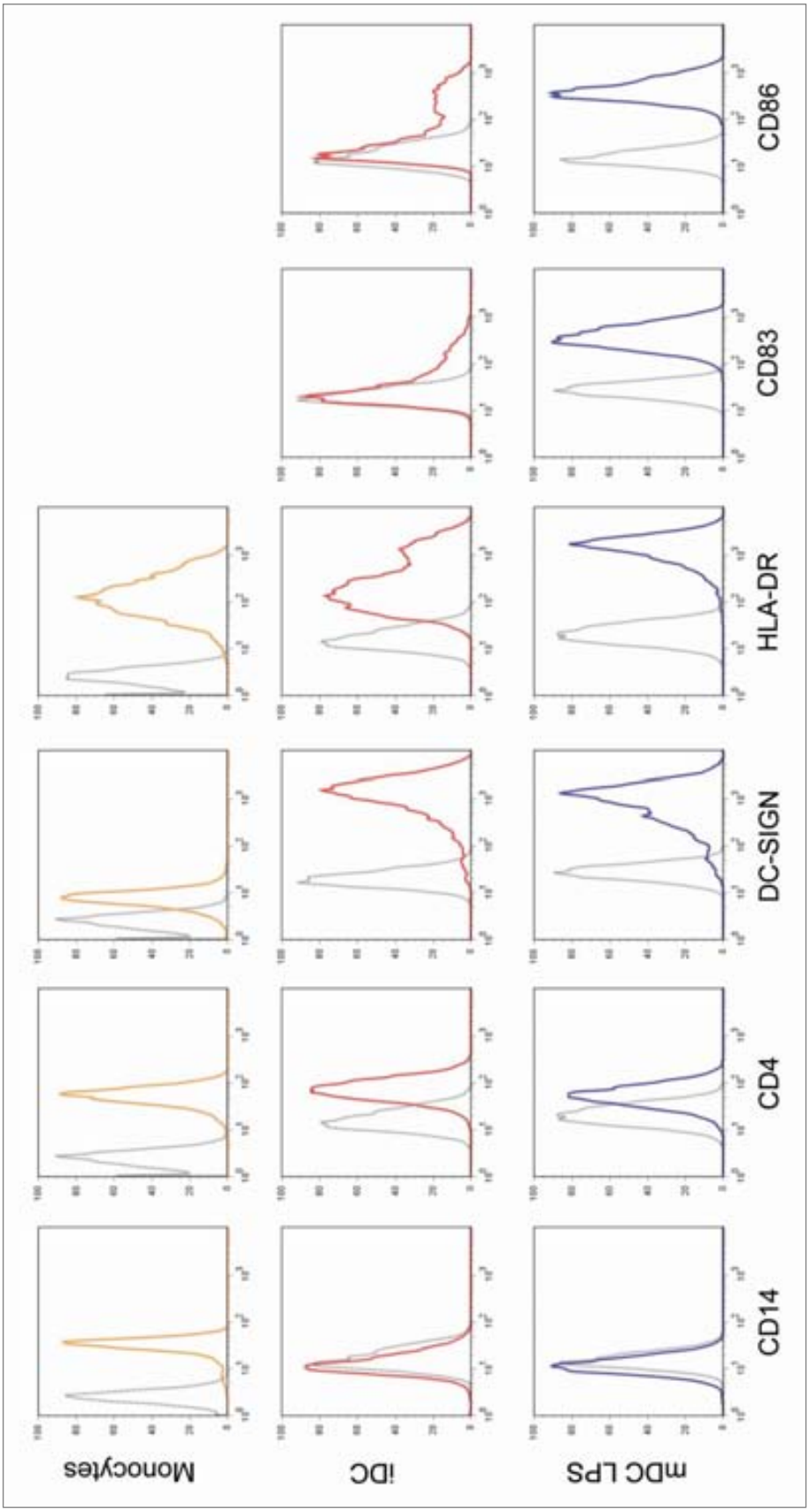


**Figure 13.** Comparative immunophenotype of monocytes, iDC and mDC LPS with surface markers CD14, CD4, DC-SIGN, HLA-DR, CD83 and CD86.

The pGag-eGFP was obtained through the NIH AIDS Research and Reference Reagent Program from Dr. Marilyn Resh [235-240] to generate viral-like particles $VLP_{HIV\text{-}Gag\text{-}eGFP}$ containing HIV-1 Gag-eGFP fusion protein. The plasmids pNL4-3 and pEGFP/Vpr, containing the Vpr viral protein fused to eGFP, were used to produce the $HIV_{NL4\text{-}3\text{-}Vpr\text{-}eGFP}$, as described previously [241].

### 4.1 Plasmid constructions

For antigen presenting assays, both pNL4-3 and pNL4-3ΔIN were modified to express the optimal epitope (SLYNTVATL) [242] and escape epitope (SLFNTIAVL) [243] for $p17^{gag}$ 77-85 (SL9 epitope) restricted by HLA-A*02, since wild-type plasmids codified for a partial escape mutant (SLYNTIAVL) [243] (Fig. 14).

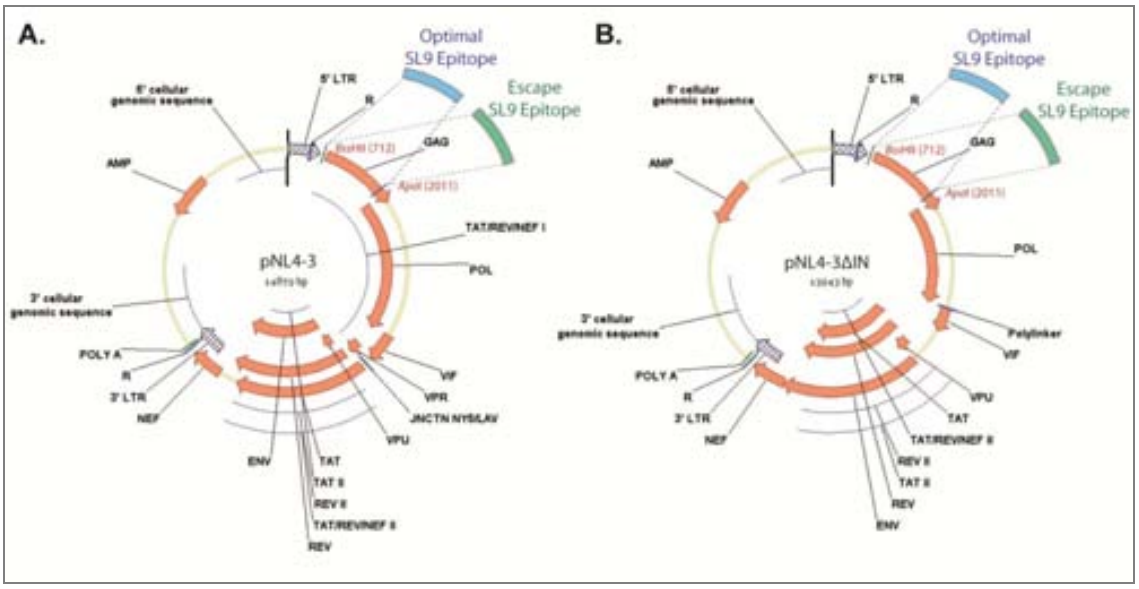


**Figure 14.** Map representation of plasmid constructions **(A)** pNL4-3 and **(B)** pNL4-3ΔIN expressing the optimal or the escape variants for $p17^{Gag}$ 77-85 (SL9 epitope) restricted by HLA-A*02.

The optimal epitope (SLYNTVATL) for $p17^{gag}$ 77-85 (SL9 epitope) restricted by HLA-A*02 was obtained from a primary viral isolate. Viral RNA was isolated with the QIAamp Viral RNA kit (QIAGEN) following manufacturer's instructions and a fragment corresponding to 704 pb to 2021 pb was amplified through Reverse Transcription-PCR using the specific primers BssHII U22 and p24ApaI (Table 2) and the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen). The program used in the PCR was the following: 1 cycle of 30 minutes at 55ºC, 2 minutes at 94ºC;

40 cycles of 1 minute at 94ºC, 1 minute at 55ºC and 1,5 minutes at 68ºC; and finally 1 cycle of 5 minutes at 68ºC. The PCR product was analyzed by 1% agarose gel electrophoresis. The PCR product was purified with QIAquick PCR Purification Kit (QIAGEN) as described by the manufacturer. A total of 80 ng of PCR product were digested using the FastDigest Enzymes *BssHII* (712pb) and *ApaI* (2011pb) (Fermentas), according to manufacturer's instructions.

The escape epitope (SLFNTIAVL) for $p17^{gag}$ 77-85 (SL9 epitope) restricted by HLA-A*02 was obtained from the vector p83-2 TriV/SL9 (containing the 5'-half-HIV-1 genome plasmid) constructed with site-directed mutagenesis by Dr. J.G. Prado. A total of 2 µg of pNL4-3, pNL4-3ΔIN and p83-2 TriV/SL9 were cut using the FastDigest Enzymes *BssHII* (712pb) and *ApaI* (2011pb), in the presence of FastDigest *Alkaline Phosphatase* (Fermentas) to avoid plasmid recircularization, according to manufacturer's instructions. After digestion, DNA fragments corresponding to bands of 13580 pb for pNL4-3, 12644 pb for pNL4-3ΔIN and 1299 pb for p83-2 TriV/SL9 were extracted from 1% agarose gel electroforesis using QIAEX II Gel Extraction Kit (QIAGEN) following the manufacturer's instructions.

The fragments of 1299 pb containing the optimal epitope (SLYNTVATL) from the viral isolate or the escape epitope (SLFNTIAVL) from the vector p83-2 TriV/SL9 were then subcloned in the backbone of pNL4-3 (13580 pb) or pNL4-3ΔIN (12644 pb) in a 3:1 ratio of insert:vector using the T4 DNA Ligase (New England BioLabs) with an overnight incubation at 16ºC. Subsequently, cloning reaction constructs were transformed into One Shot Stbl3 Chemically Competent *E. coli* (Invitrogen), according the manufacturer's instructions, spread on LB plates containing 100 µg/ml Ampicilin (SigmaAldrich) and incubated overnight at 37ºC. Individual colonies were selected and grown for 2 hours at 37ºC in LB medium containing 100 µg/ml Amplicilin. To confirm the presence of the insert into the vector, colonies were directly analyzed by PCR, without prior DNA extraction, using the specific primers BssHII U22 and p24ApaI (Table 2) and the AmpliTaq Gold DNA Polymerase (Applied Biosystems) with 4% DMSO. The program used in the PCR was the following: 1 cycle of 10 minutes at 95ºC; 25 cycles of 1 minute at 94ºC, 1 minute at 55ºC and 1,5 minutes at 72ºC; and finally 1 cycle of 10 minutes at 72ºC. The PCR products were analyzed by 1% agarose gel electrophoresis and positive clones were selected and grown overnight at 37ºC in LB medium containing 100 µg/ml Amplicilin. Then, plasmids from clones with positive PCR screening were purified using the QIAprep Spin Miniprep Kit (QIAGEN), and sequenced using the specific primers 5OPF1, sec3F1, sec5F1 and sec6F1 (Table 2).

Sequencing was performed by Macrogen Inc. and analyzed using the Sequencher v4.7 (Gene Codes Corporation). Clones of pNL4-3 expressing the optimal or the escape epitope (pNL4-3 SL9 Optimal and pNL4-3 SL9 Escape, respectively) and clones of pNL4-3ΔIN expressing the optimal or the escape epitope (pNL4-3ΔIN SL9 Optimal and pNL4-3ΔIN SL9 Escape, respectively) were selected and glycerolates of each clone were stored at -80ºC to preserve the transformed cells.

**Table 2.** Primers used for PCR amplificaction and sequencing to construct the HIV-1 plasmids pNL4-3 and pNL4-3ΔIN expressing the optimal or the escape epitope for $p17^{Gag}$ 77-85 (SL9 epitope) restricted by HLA-A*02 (pNL4-3 SL9 Optimal, pNL4-3 SL9 Escape, pNL4-3ΔIN SL9 Optimal and pNL4-3ΔIN SL9 Escape plasmids).

| Primer name | Sequence 5' to 3' | Strand | HXB2 location |
|---|---|---|---|
| BssHII U22 | TGCTGAAGCGCGCACGGCAAGA | Forward | 704-725 |
| p24ApaI | TTTTTCCTAGGGGCCCT | Reverse | 2021-2005 |
| 5OP F1 | CTAGCAGTGGCGCCCGAACA | Forward | 629-648 |
| Sec3F1 | GACACCAAGGAAGCCTTAG | Forward | 1075-1093 |
| Sec5F1 | GGAACAAATAGCATGGATGAC | Forward | 1521-1541 |
| Sec6F1 | TTTTCCACATTTCCAACAGCC | Reverse | 2043-2023 |

## 5. Viral stocks

### 5.1 Obtained by transfection

The $HIV_{NL4-3}$, $HIV_{NL4-3\Delta IN}$, $HIV_{NFN-SX}$, $HIV_{NL4-3\Delta ENV}$, $VLP_{HIV-Gag-eGFP}$ stocks were generated by transfecting HEK-293T cells seeded in 75 $cm^2$ flasks with 30-36 µg of the corresponding proviral construct (pNL4-3, pNL4-3ΔIN, pNFN-SX, pNL4-3.Luc.$R^-$.$E^-$, pGag-eGFP, respectively) using CalPhos Transfection Kit (Clontech), following the manufacturer's instructions. To obtain the $HIV_{NL4-3-Vpr-eGFP}$, HEK-293T cells were co-transfected by the plasmids pNL4-3 and pEGFP/Vpr, whereas the $HIV_{NL4-3-Cherry}$ was generated following co-transfection of pCHIV and pCHIV mCherry in a 1:1 ratio. Forty-

eight hours after transfection using CalPhos Transfection Kit (Clontech), supernatants containing virus were collected, filtered (Millex HV, 0.45 μm; Millipore) and frozen at –80ºC until use (Fig. 15 A).

Viral stocks of $HIV_{NL4\text{-}3\ SL9\ Optimal}$, $HIV_{NL4\text{-}3\ SL9\ Escape}$, $HIV_{NL4\text{-}3\Delta IN\ SL9\ Optimal}$, $HIV_{NL4\text{-}3\Delta IN\ SL9\ Escape}$, and $HIV_{NL4\text{-}3Ren}$ were produced as explained above, and concentrated using the Lenti-X Concentrator kit (Clontech) as described by the manufacturer before storage at -80ºC.

For viral fusion assays, $HIV_{NL4\text{-}3}$ and $HIV_{NL4\text{-}3\Delta IN}$ virions containing the β-lactamase-Vpr (BlaM-Vpr) chimera were produced as previously described [234]. In brief, $1{,}5x10^7$ HEK-293T cells were seeded in a 175 $cm^2$ flasks. The day after, cells were co-transfected by 60 μg of pNL4-3 or pNL4-3ΔIN proviral DNA, 20 μg of pCMV-BlaM-Vpr, and 10 μg of pAdVAntage vectors with calcium phosphate CalPhos Transfection Kit, according to manufacturer's instructions. BlaM-Vpr is specifically incorporated into the virion through the Vpr interaction with the p6 component of the Gag polyprotein. Supernatants containing virus were collected 48 hours later, filtered (Millex HV, 0.45 μm; Millipore), ultracentrifuged at 72,000*g* for 90 minutes at 4ºC and frozen at –80ºC until use.

The $p24^{Gag}$ content of all viral stocks was measured using an ELISA (PerkinElmer). Titers of all viruses were determined using the TZM-bl indicator cell line, that enables simple and quantitative analysis of HIV-1 using luciferase as a reporter [244]. Cells were assayed for luciferase activity 48 hours after infection (Bright-Glo Luciferase Assay System; Promega) in a Fluoroskan Ascent FL Luminometer (Thermo Scientific).

### 5.2 Obtained from primary cells

To generate $CD4^+$ T-cell derived HIV-1 viral stocks, enriched $CD4^+$ T cells stimulated with 0.5 μg/ml low-dose PHA, 5 μg/mL high-dose PHA or plate-bound anti-CD3 mAb OKT3 for 72 hours (Fig. 12 B) were infected with a multiplicity of infection (MOI) of 0.02 of $HIV_{NL4\text{-}3}$ during 3 hours at 37ºC in 5% $CO_2$. Then, cells were extensively wash with PBS and resuspended to a final concentration of $10^6$ cells/ml in RPMI-1640 with 10% heat-inactivated FBS supplemented with 100 U/ml of IL-2. Concentration of $p24^{Gag}$ in the supernatant was monitored by an ELISA (PerkinElmer) and when it was at least $10^2$ ng $p24^{Gag}$/ml, viral stocks were harvested, filtered (Millex HV, 0.45 μm; Millipore) and stored at -80ºC until use (Fig. 15 B).

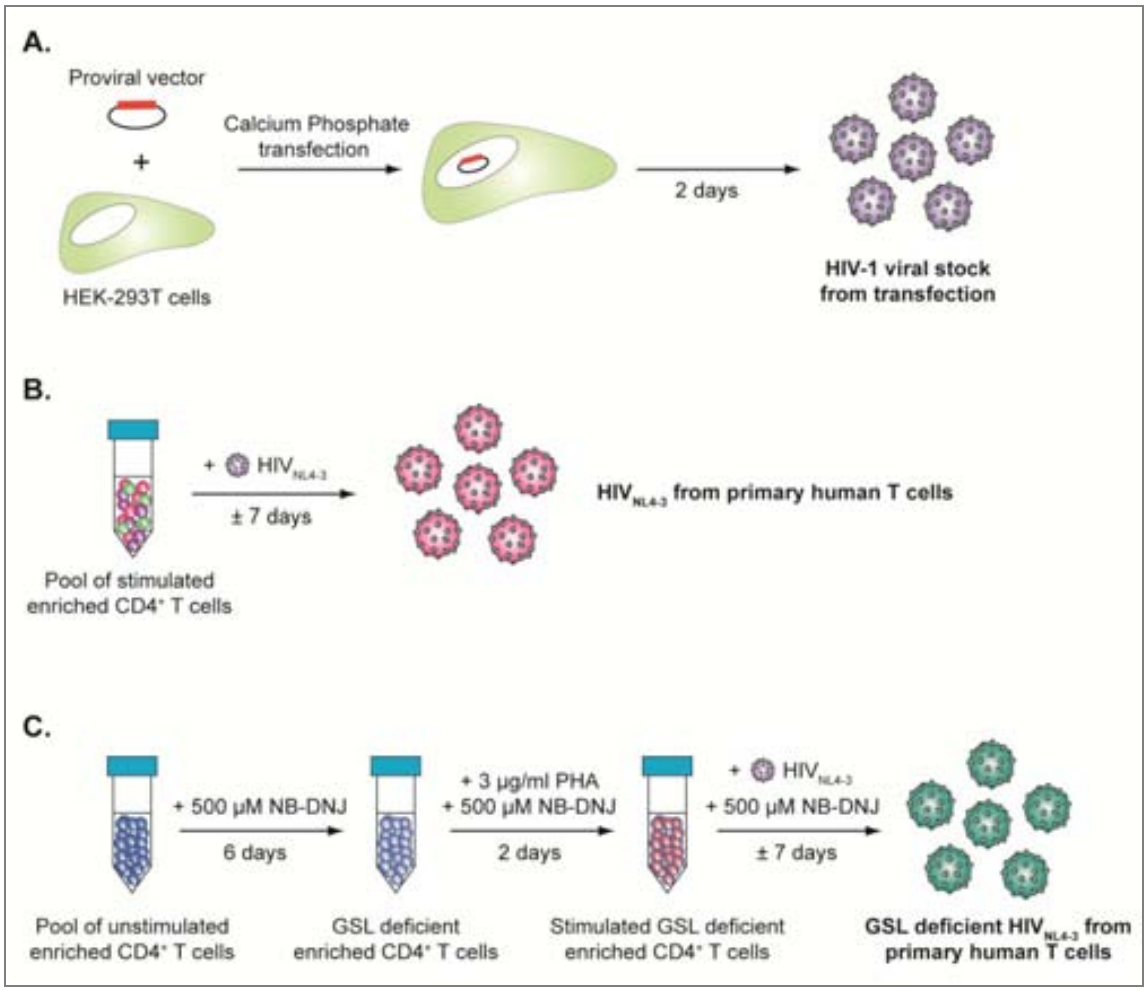


**Figure 15.** Schematic representation of the different protocols followed to obtain **(A)** HIV-1 stocks from calcium phosphate transfection of HEK-293T cells, **(B)** $HIV_{NL4-3}$ from primary human T cells, and **(C)** GSL-deficient $HIV_{NL4-3}$ from primary human T cells. *GSL:* Glycosphingolipid. *NB-DNJ:* N-Butyldeoxynojirimycin Hydrochloride.

To produce GSL-deficient $HIV_{NL4-3}$, non-activated enriched $CD4^+$ T cells were kept in the presence or absence of 500 µM of N-Butyldeoxynojirimycin Hydrochloride (NB-DNJ; Calbiochem, Merck Chemicals Ltd.) and 10 U/ml of IL-2 for six days. Cells were then stimulated with 3 µg/ml of PHA for two days in the presence of 500 µM of NB-DNJ. Next, GSL-deficient enriched $CD4^+$ T cells were infected with $HIV_{NL4-3}$ at a MOI = 0.02 during 3 hours at 37ºC in 5% $CO_2$. Cells were then extensively wash with PBS and resuspended to a final concentration of $10^6$ cells/ml in RPMI-1640 with 10% heat-inactivated FBS supplemented with 100 U/ml of IL-2 and 500 µM of NB-DNJ. Simultaneously, a control $HIV_{NL4-3}$ stock was generated in enriched $CD4^+$ T cells under the same culture conditions but in the absence of NB-DNJ treatment. Virus stocks were

harvested, filtered and stored at -80°C until use when the concentration of $p24^{Gag}$ was at least $10^2$ ng $p24^{Gag}$/ml (Fig. 15 C).

The $p24^{Gag}$ content of all viral stocks was measured by an ELISA assay (PerkinElmer) and titers of all viruses were determined by using TZM-bl indicator cell line as described elsewhere [244].



## 6. HIV-1 virion-based fusion assay

The viral fusion assay was performed as previously described [234] (Fig. 16). Briefly, 5 x $10^5$ Jurkat T cells were infected with 400 ng $p24^{Gag}$ of $HIV_{NL4-3}$ or $HIV_{NL4-3\Delta IN}$ containing BlaM-Vpr for 4 hours (spinoculation at 600*g* for 90 minutes at 22ºC followed by incubation for 2.5 hours at 37ºC in 5% $CO_2$) in the presence or absence of 5 µg/ml C34 fusion inhibitor (NIH AIDS Research and Reference Reagent Program) [245]. Cells were then washed in $CO_2$-independent medium (Gibco; Invitrogen) to remove free virions and loaded with 1 mM CCF2-AM dye (Invitrogen) for 1 hour at room temperature as described by the manufacturer. After two washes with development medium ($CO_2$-independent medium supplemented with 10% heat-inactivated FBS), the BlaM cleavage of CCF2 reaction was allowed to proceed in the dark for 16 hours at room temperature in 200 µl of development medium. Finally, cells were washed in PBS and fixed in a 1.2% formaldehyde solution. The degradation of CCF2-AM by BlaM cleavage and its change in emission fluorescence was measured by flow cytometry using an LSR II (Becton Dickinson). Data were collected with FACSDiva software (Becton Dickinson) and analyzed with FlowJo software (Tree Star).

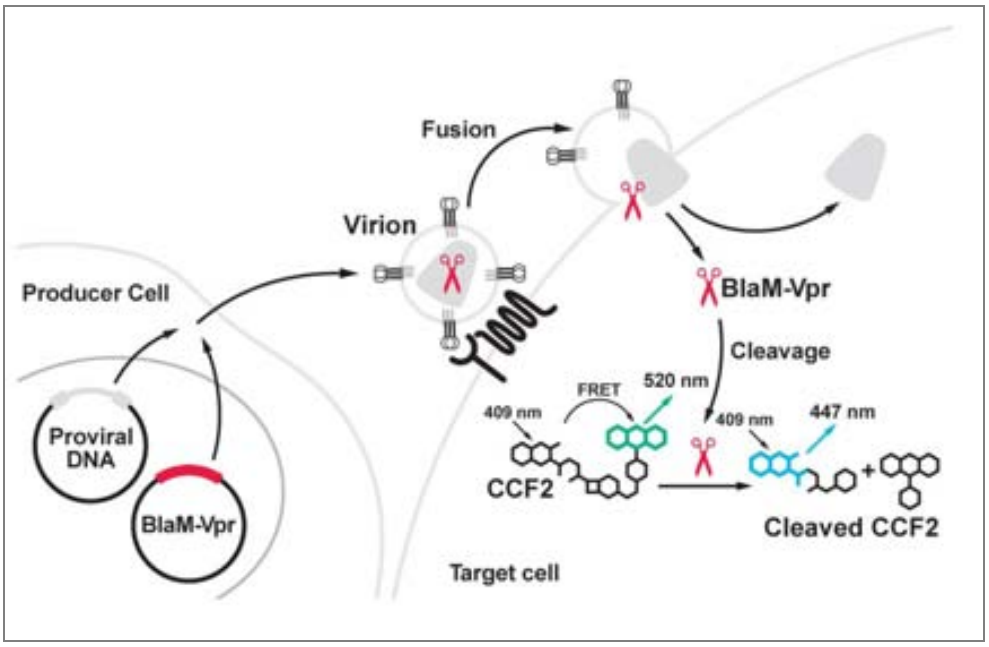


**Figure 16.** *Reproduced from Cavrois, M.C., et al., 2002* [234]. Schematic overview of the HIV-1 virion based fusion assay, developed by *Cavrois M et al* [234]. The expression plasmids pNL4-3 or pNL4-3ΔIN, (encoding HIV provirus $HIV_{NL4-3}$ or $HIV_{NL4-3\Delta IN}$ respectively), pCMV–BlaM-Vpr (codifying for BlaM-Vpr chimera) and the pAdVAntage vector are co-expressed in HEK-293T producer cells. Target cells are infected with BlaM-Vpr virions and then loaded with CCF2-AM dye, which diffuses passively across the cell membrane. Inside the cell, CCF2 is de-esterified and trapped because of its polyanionic properties. CCF2 contains a cephalosporin ring linking a 7-hydroxycoumarin to fluorescein. In this configuration, excitation of the coumarin at 409 nm leads to fluorescent resonance energy transfer (FRET) to the fluorescein moiety, producing a green emission (520 nm). If the β-lactam ring in the cephalosporin moiety is cleaved by BlaM, FRET is blocked, and excitation of the coumarin yields a blue emission (447 nm) [246]. Thus, the changes in the pattern of CCF2 fluorescence reflect the presence of BlaM, introduced by virion fusion, within the target cells. If viral fusion occurs, BlaM cleaves the β-lactam ring in CCF2, changing its fluorescence emission spectrum from green (520 nm) to blue (447 nm).

## 7. DC viral capture assay

To assess the ability of DC to capture HIV-1, 2,5 x $10^5$ DC were incubated at 37ºC in 5% $CO_2$ with HIV-1 (50 – 90 ng $p24^{Gag}$) at a final concentration of 1 x $10^6$ cells/ml. Incubation times as well as the amount of virus differed depending on the experiment. After viral incubation, DC were washed thoroughly with PBS to remove unbound particles. Cells were then lysed with 0.5% Triton X-100 at final concentration of 5 x $10^5$ cells/ml and intracellular $p24^{Gag}$ antigen content was measured by a specific ELISA assay (PerkinElmer) (Fig. 17).

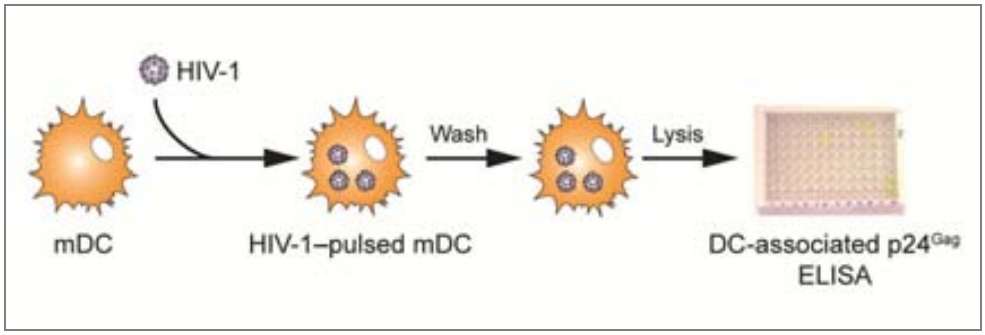


**Figure 17.** Schematic representation of the experimental procedure used in DC viral capture assays.

The exact experimental conditions for each assay are listed below.

- To evaluate the effect of DC maturation state and activation stimulus in the capacity of DC to uptake HIV-1, iDC, mDC ITIP or mDC LPS were incubated for 6 hours with 50 ng $p24^{Gag}$ of $HIV_{NL4-3}$ or $HIV_{NL4-3\Delta IN}$ obtained from transfection of HEK-293T cells. During viral incubation, some iDC were matured with ITIP (iDC+ITIP), others with LPS (iDC+LPS), and the rest were immature (iDC).

- To study the potential role of GSL for HIV-1 capture by DC, mDC LPS were pre-incubated with or without 5 mM of soluble GM3 carbohydrate head group (Carbosynth) or Lactose (SigmaAldrich) for 30 minutes at room temperature. Cells were then exposed to 90 ng $p24^{Gag}$ $HIV_{NL4-3}$ obtained from primary $CD4^+$ T cells for 4 hours. The compounds tested were maintained at a constant concentration of 10 mM during viral incubation.

- To confirm the importance of viral gangliosides for DC capture, mDC LPS were exposed for 4 hours to 50ng $p24^{Gag}$ $HIV_{NL4-3}$ or GSL-deficient $HIV_{NL4-3}$ (derived from NB-DNJ treated cells) obtained from primary $CD4^+$ T cells**.**

- To test whether Siglec-1 is a DC receptor that mediates viral capture, iDC, mDC ITIP or mDC LPS were pre-incubated at 16ºC for 30 minutes with the mAb anti-Siglec-1 (10 µg/ml) or isotype control (10 µg/ml) (Table 3) or with mannan (500 µg/ml; SigmaAldrich). Subsequently, DC were pulsed for 5 hours with 50 ng $p24^{Gag}$ $HIV_{NL4-3}$ obtained from transfection of HEK-293T cells. In parallel, untreated DC equally pulsed with $HIV_{NL4-3}$ were incubated for 30 minutes at 16ºC with the mAb anti-Siglec-1 (10 µg/ml) or isotype control (10 µg/ml) or with mannan (500 µg/ml) right after viral capture.

**Table 3.** Monoclonal antibody (mAb) and reagents used in the DC viral capture assays.

| Molecule name | Clone | Source |
|---|---|---|
| Isotype control | 107.3 | BD Biosciences |
| Siglec-1 (Sialoadhesin) | HSn 7D2 | abcam |

## 8. DC-mediated HIV-1 *trans*-infection assays

In order to evaluate the HIV-1 transmission efficiencies of different DC subsets, the $CD4^+$ T lymphocytes and the TZM-bl cell line were used as susceptible cells. The use of these target cells involved different strategies to detect viral infection. In the *trans*-infection assays performed with primary $CD4^+$ T cells and wild-type virus isolates, HIV-1 infection was detected by FACS using intracellular staining with specific mAb. The experiments with primary $CD4^+$ T lymphocytes were also performed using an infectious reporter virus, the $HIV_{NL4-3Ren}$, which expresses the Renilla luciferase protein and allows evaluation of HIV-1 infection by detection of luciferase activity. Conversely, the TZM-bl is a reporter cell line itself and was used with wild-type virus. The TZM-bl cell line also enabled quantification of viral infection by luminescence, since it contains an integrate copy of the luciferase gene under the control of the HIV-1 promoter.

### 8.1 mDC-mediated HIV-1 *trans*-infection to primary $CD4^+$ T cells

#### *8.1.1 Evaluation of GSL in viral transmission*

mDC LPS were pre-incubated with or without 5 mM of soluble GM3 carbohydrate head group for 30 minutes at room temperature and then maintained at a constant concentration of 10 mM during the rest of the experiment. Next, mDC LPS were exposed to $HIV_{NL4-3}$, obtained from primary $CD4^+$ T cells, at a MOI = 0.1 for 4 hours at 37 ºC in 5% $CO_2$. Alternatively, non-GM3 treated mDC LPS were incubated for 4 hours at 37 ºC in 5% $CO_2$ with a MOI = 0.1 of $HIV_{NL4-3}$ or GSL-deficient $HIV_{NL4-3}$ (derived from NB-DNJ treated cells) obtained from primary $CD4^+$ T cells. HIV-1–pulsed mDC LPS were subsequently co-cultured in duplicate with PHA-activated primary $CD4^+$ T cells, at a ratio of 1 x $10^5$ DC : 1.5 x $10^5$ $CD4^+$ T cells, for 48 hours on a 96 well U-bottom plate

without removal of unbound viral particles. Co-cultures were performed in the presence or in the absence of 0,5 µM of the protease inhibitor saquinavir (SQV), to discriminate single-round infection due to *trans*-infection from re-infection events. To detect the possible cell-free virus infection of activated $CD4^+$ T cells, an equal MOI was added directly to control wells lacking mDC LPS. Infection of activated primary $CD4^+$ T cells was detected with flow cytometry, measuring the intracellular $p24^{Gag}$ content within the CD2-positive CD11c-negative population of $CD4^+$ T cells staining the molecules $p24^{Gag}$, CD2 and CD11c with the mAb listed in Table 4. Samples were acquired on an LSR II flow cytometer (BD Biosciences) and cells were analyzed within the CD2-positive CD11c-negative singlet gate using the FlowJo software (Tree Star) (Fig. 18).

**Table 4.** Monoclonal antibodies (mAb) used to assess the DC-mediated *trans*-infection to stimulated primary $CD4^+$ T cells of $CD4^+$ T cell derived $HIV_{NL4-3}$.

| Molecule name | Clone | Fluorochrome | Source |
|---|---|---|---|
| CD2 | RPA-2.10 | PerCP-Cy5.5 | BD Pharmingen |
| CD11c | Bu15 | APC/Cy7 | BioLegend |
| $p24^{Gag}$ (HIV-1 proteins 55, 39, 33 and 24 kDa of core antigen) | FH190-1-1 (KC57) | FITC | Beckman Coulter |

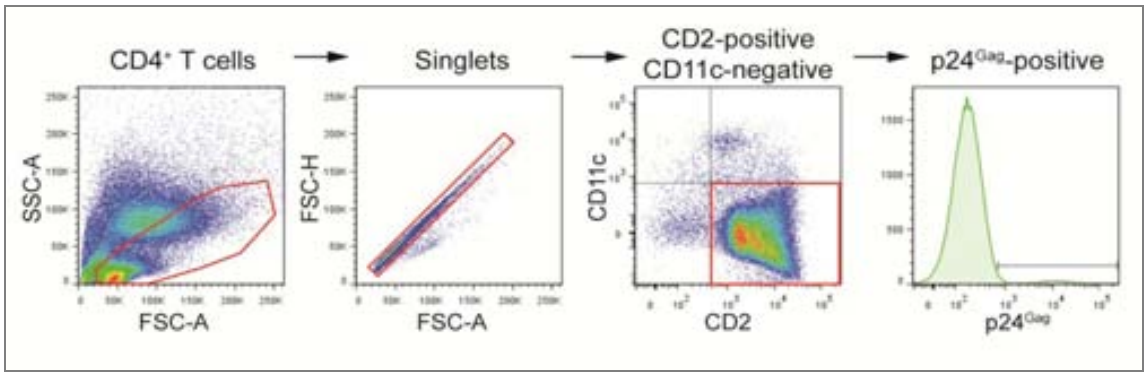


**Figure 18.** Strategy of gating to evaluate the mDC-mediated *trans*-infection of HIV-1 to primary $CD4^+$ T cells by flow cytometry.

### *8.1.2 Evaluation of adhesion molecules and antigen recognition in viral transmission across the infectious synapse*

Transmission of HIV-1 from mDC to $CD4^+$ T cells was assessed by co-culturing $1x10^5$ virus-pulsed mDC with $1.5x10^5$ autologous or allogeneic non-activated primary $CD4^+$ T cells for 48 hours at 37ºC in 5% $CO_2$. First, mDC were incubated with $HIV_{NL4-3Ren}$, obtained from transfection of HEK-293T cells, at a MOI = 0.1 (based on HIV-1 titration in TZM-bl cells) for 5 hours at 37ºC in 5% $CO_2$. Cells were then extensively washed with PBS to remove uncaptured viral particles. Subsequently, mDC and $CD4^+$ T cells were separately pre-incubated for 30 minutes at room temperature in the presence or absence of different mAb and reagents (Table 5): anti-LFA-1 (10 µg/ml), anti-ICAM-1 (10 µg/ml), anti-ICAM-3 (10 µg/ml), anti-CD4 (10 µg/ml), isotype control Ab (10 µg/ml), Staphylococcal enterotoxin A from *Staphylococcus aureus* (SEA; 10 µg/ml) and cytochalasin D (5 µM), or with azidothymidine (AZT; 5 µM), or SQV (0.5 µM). Then, HIV-1–pulsed mDC and autologous or allogeneic non-activated primary $CD4^+$ T cells were co-cultured in a final volume of 200 µl of RPMI-1640 containing 10% FBS on a 96 well U-bottom plate in the presence of the indicated blocking reagents (Fig. 19). After 48 hours of co-culture, luciferase activity was assayed with the Renilla-Glo Luciferase Assay System (Promega), according to manufacturer's instructions, using a 96-well plate Fluoroskan Ascent FL Luminometer. To specifically show the infection of $CD4^+$ T cells in co-cultures, background values based on HIV-1–exposed mDC cultured alone were subtracted for each co-culture condition, although luminescence values of HIV-1–pulsed mDC were comparable to unpulsed mDC.

**Table 5.** Monoclonal antibodies (mAb) and reagents used in the blocking assays to prevent viral transmission between mDC and $CD4^+$ T cells.

| Molecule name | Clone | Source |
|---|---|---|
| CD4 (Leu3a) | SK3 | BD Biosciences |
| ICAM-1 (CD54) | Rm3a5 | Dr. R. Vilella, Hospital Clínic, Barcelona |
| ICAM-3 (CD50 domain 1) | 101.1D2 | Dr. R. Vilella, Hospital Clínic, Barcelona |
| Isotype control Ab | P3.6.2.8.1 | eBioscience |

| | | |
|---|---|---|
| LFA-1 (CD18) | 68.5a5 | Dr. R. Vilella, Hospital Clínic, Barcelona |
| Azidothymidine (AZT) | - | NIH AIDS Research and Reference Reagent Program |
| Cytochalasin D | - | SigmaAldrich |
| Saquinavir (SQV) | - | NIH AIDS Research and Reference Reagent Program |
| Staphylococcal enterotoxin A from *Staphylococcus aureus* (SEA) | - | SigmaAldrich |

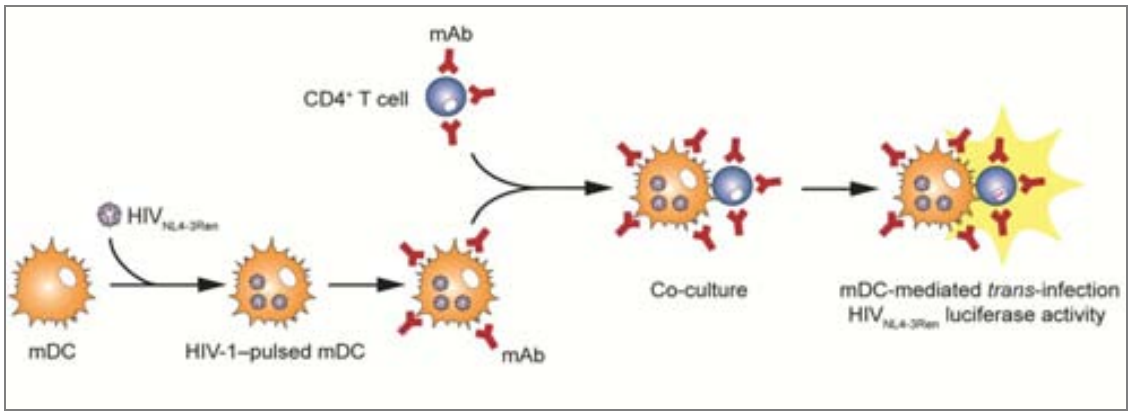


**Figure 19.** Schematic representation of the experimental procedure used for quantification of mDC-mediated *trans*-infection of the infectious reporter virus HIV$_{NL4-3Ren}$ to non-activated CD4$^+$ T cells in the presence of several mAb or reagents.

## 8.2 DC-mediated *trans*-infection of HIV-1 to TZM-bl reporter cell line

### *8.2.1 Evaluation of DC maturation state and activation stimulus in viral transmission*

A total of 2.5 x $10^5$ iDC, mDC ITIP or mDC LPS was incubated at 37°C for 6 hours with 50 ng p24$^{Gag}$ of HIV$_{NL4-3}$ or HIV$_{NL4-3\Delta IN}$, obtained from transfection of HEK-293T cells, at a final concentration of 1 x $10^6$ cells/ml. During viral pulse, some iDC were matured with ITIP (iDC+ITIP), others were matured with LPS (iDC+LPS), and the rest were immature (iDC). After incubation, cells were extensively washed with PBS to

remove uncaptured viral particles. HIV-1–pulsed mDC were extensively washed with PBS to remove uncaptured virions and co-cultured in quatriplicate with the TZM-bl reporter cell line at a ratio of $10^4$ DC : $10^4$ TZM-bl cells at a final volume of 100 µl of RPMI-1640 containing 10% FBS (Fig. 20). Cells were assayed for luciferase activity after 48 hours of co-culture (Bright-Glo Luciferase Assay System; Promega), according to manufacturer's instructrions, using a Fluoroskan Ascent FL Luminometer. Background values consisting of non-HIV-1 pulsed co-cultures were substracted for each sample.

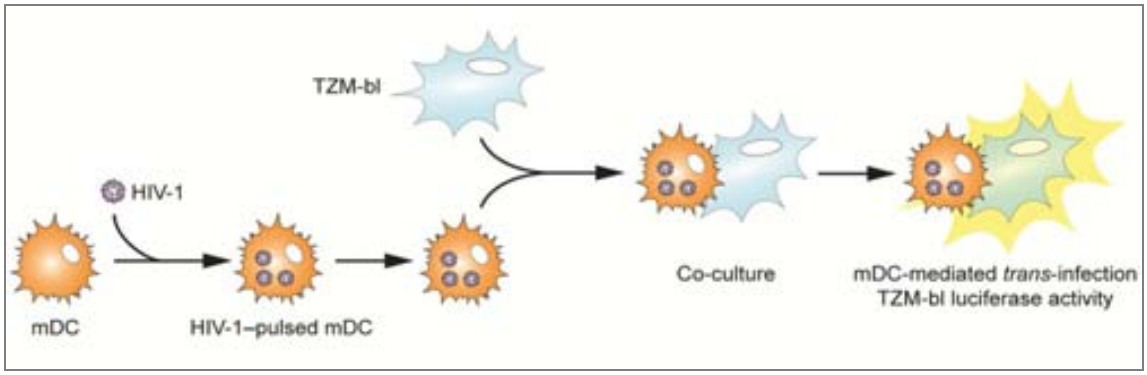


**Figure 20.** Schematic representation of the experimental procedure used for quantification of mDC-mediated *trans*-infection of HIV-1 to reporter cell line TZM-bl.

### *8.2.2 Evaluation of GSL in viral transmission*

mDC LPS were pre-incubated with or without 5 mM of soluble GM3 carbohydrate head group for 30 minutes at room temperature and then maintained at a constant concentration of 10 mM during the rest of the experiment. Then, 2.5 x $10^5$ mDC LPS were exposed to $HIV_{NL4-3}$, obtained from primary $CD4^+$ T cells, at a MOI = 0.1 for 4 hours at 37ºC in 5% $CO_2$. Alternatively, non-GM3 treated mDC LPS were incubated for 4 hours at 37 ºC in 5% $CO_2$ with a MOI = 0.1 of $HIV_{NL4-3}$ or GSL-deficient $HIV_{NL4-3}$ (derived from NB-DNJ treated cells) obtained from primary $CD4^+$ T cells. HIV-1–pulsed mDC were extensively washed with PBS to remove uncaptured virions and co-cultured in quatriplicate with the TZM-bl reporter cell line at a ratio of $10^4$ DC : $10^4$ TZM-bl cells at a final volume of 100 µl of RPMI-1640 containing 10% FBS in the presence of 0,5 µM of protease inhibitor SQV (Fig. 20). Cells were assayed for luciferase activity 48 hours later (BrightGLo Luciferase System; Promega), according to manufacturer's instructions, in a Fluoroskan Ascent FL luminometer. Background values consisting of non-HIV-1 exposed mDC LPS-TZM-bl co-cultures were subtracted for each sample.

### *8.2.3 Evaluation of Siglec-1 as a DC receptor that mediates HIV-1* **trans-*infection***

A total of 2.5 x $10^5$ iDC, mDC ITIP or mDC LPS were pre-incubated at 16ºC for 30 minutes with the mAb anti-Siglec-1 (10 µg/ml) or isotype control (10 µg/ml) (Table 3) or with mannan (500 µg/ml). Cells were then incubated at 37°C for 5 hours with a MOI = 0.1 of $HIV_{NL4-3}$, obtained from transfection of HEK-293T cells, at a final concentration of 1 x $10^6$ cells/ml. Then, some untreated cells were incubated at 16ºC for 30 minutes with anti-Siglec-1 (10 µg/ml) or isotype control (10 µg/ml) (Table 3) or with mannan (500 µg/ml). After viral pulse, cells were thoroughly washed with PBS to remove uncaptured viral particles. Then, $10^4$ HIV-1–pulsed DC were co-cultured in quadruplicate with the reporter cell line TZM-bl at a ratio of 1:1 per well in a 96-well plate, at a final volume of 100 µl of RPMI-1640 containing 10% FBS, in the presence of 0.5 µM of SQV when indicated (Fig. 20). Cells were assayed for luciferase activity after 48 hours of co-culture (Brighty-Glo Luciferase assay System; Promega), according to manufacturer's instructions, in a Fluoroskan Ascent FL Luminometer. Background values consisting of non-HIV-1 pulsed co-cultures were substracted for each sample.

## 9. $VLP_{HIV\text{-}Gag\text{-}eGFP}$ kinetic capture assay

At day 5 of monocyte-derived differentiation of DC, LPS was added to iDC for 2 days to obtain mDC LPS. $VLP_{HIV\text{-}Gag\text{-}eGFP}$ capture was assessed to DC at different time points after LPS addition to iDC. A total of 2x$10^5$ DC were incubated with 2500 pg of $VLP_{HIV\text{-}Gag\text{-}eGFP}$ $p24^{Gag}$ for 1 hour at 37ºC at time points 0 hours, 6 hours, 24 hours and 48 hours after addition of LPS to iDC. At same time points, DC were stained to assess cell membrane expression of CD83, CD169 and HLA-DR molecules (Table 6). Samples were acquired by FACS with a FACSCalibur Flow Cytometer (BD Bioscience) and analyzed with FlowJo software (Tree Star).

**Table 6.** Monoclonal antibodies (mAb) used to immunophenotype iDC and mDC LPS by flow cytometry.

| Molecule name | Clone | Fluorochrome | Source |
|---|---|---|---|
| CD83 | HB15e | FITC | BD Pharmingen |
| CD169 (Sialoadhesin) | 7-239 | PE | AbDSerotec |
| HLA-DR | L243 | PerCP | BD Biosciences |

## 10. Antigen presentation assays

In order to evaluate the impact of the time lag between DC maturation and antigen loading in the stimulation of HIV-1–specific T-cell clones, antigen presentation assays were designed as follows (Fig. 21). It is worth noting that, although differing in the time lag between maturation and viral loading as well as in the exposure time to maturation stimuli, all DC conditions underwent the same exposure time to virus for HLA-I or HLA-II antigen presentation assays.

### 10.1 HLA-I antigen presentation assays

For HLA-I antigen presentation experiments, monocytes from HLA-A*02$^{+}$ donors were used to generate DC. iDC, mDC ITIP and mDC LPS were exposed for 24 hours to the viruses (500 ng $p24^{Gag}$/ml per 1 x $10^6$ cells) at 37°C in 5% $CO_2$ in culture medium with IL-4, GM-CSF, 5 µM AZT, and 1.2 µM Nevirapine (NVP) (AZT and NVP both from SigmaAldrich). During viral incubation, iDC were simultaneously matured with ITIP (iDC+ITIP), LPS (iDC+LPS), or remained unstimulated (iDC). Cells were then extensively washed with PBS to remove unbound viruses and co-cultured for 16-18 hours with SL9-2 or EM40-F21 CTL clones in ELISPOT plates (Fig. 21). T-cell activation was monitored using the IFN-γ ELISPOT assay as previously described [104]. Negative controls consisting of non–HIV-1–exposed DC–CD8$^{+}$ T-cell clone co-cultures were performed to discard non-specific activation of the T-cell clone. As a positive control, DC were loaded with 0.1 µg/ml of cognate peptide.

### 10.2 HLA-II antigen presentation assays

For HLA-II antigen presentation experiments, monocytes from HLA-DRß*04 or HLA-DRß*04/HLA-DRß*01 donors were used to generate DC. iDC, mDC ITIP and mDC LPS were exposed for 6 hours to the viruses (285 ng $p24^{Gag}$/ml per 1 x $10^6$ cells) at 37°C in 5% $CO_2$ in culture medium with 5 µM AZT and 1.2 µM NVP. Cells were then extensively washed with PBS to remove unbound viruses and cultured overnight in medium with IL-4, GM-CSF, AZT, and NVP. During viral incubation, iDC were simultaneously matured with ITIP (iDC+ITIP), LPS (iDC+LPS), or remained unstimulated (iDC). Cells were then washed with PBS and co-cultured for 16-18 hours with F12 or N2 $CD4^+$ T-cell clones in ELISPOT plates (Fig. 21). T-cell activation was monitored using an IFN-γ ELISPOT assay. Negative controls consisting of non–HIV-1–exposed DC–$CD4^+$ T-cell clone co-cultures were performed to discard non-specific activation of the T-cell clone. As a positive control, DC were loaded with 0.1 µg/ml of cognate peptide.

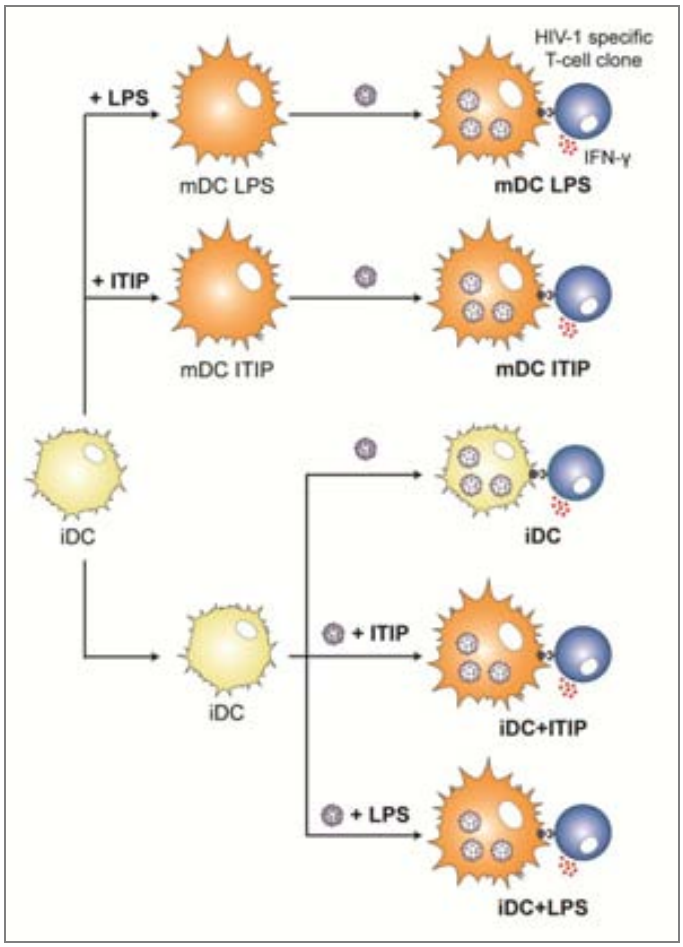


**Figure 21.** Schematic representation of the protocol for HLA-I and HLA-II antigen presentation assays.

### 10.3 IFN-γ ELISPOT assay

The IFN-γ ELISPOT assay procedure was performed according to the protocol of Dr. Moris laboratory, in the INSERM, Unité Mixte de Recherche Scientifique 945, Infection et Immunité, Université Pierre et Marie Curie in Paris (France), and as previously described [104]. In brief, hydrophobic PVDF plates (MSIPN4550, Millipore) were coated with the capture antibody by washing with 100 µl/well PBS and overnight incubation at 4ºC incubating with 2 µg/ml of unconjugated mouse anti-human IFN-γ mAb (clone 1-D1K; Mabtech) in PBS. For saturation, plates were washed six times with PBS and incubated with 100 µl/well PBS 10% FBS for 2 hours at room temperature or overnight at 4ºC. Then, liquid was discarded from wells and ELISPOT assay was set up by co-culturing DC with HIV-1–specific T-cell clones for 18 hours at 37ºC in 5% $CO_2$ in humidified incubator without perturbing the co-cultures. After incubation, supernatants were removed from wells and plate was washed twice with PBS, three times with PBS 0.05% Tween 20 (SigmaAldrich), and twice again with PBS. Next, detection of IFN-γ secretion was performed by incubating the plate 2-3 hours at room temperature or overnight at 4ºC with 1 µg/ml of biotinylated mouse anti-human IFN-γ mAb (clone 7-B6-1; Mabtech) in PBS 0.5% BSA (SigmaAldrich). Then, plate was washed twice with PBS, three times with PBS 0.05% Tween 20, and twice again with PBS to avoid bubbles; and incubated with 0.5 U/ml of Streptavidin Alkaline Phosphatase conjugate (Roche) in PBS 0.05% BSA for 45 minutes to 1.5 hours at room temperature. Plate was twice with PBS, three times with PBS 0.05% Tween 20 (SigmaAldrich), and three time with PBS to avoid bubbles. Finally, plate was developed with the alkaline phophatase substrate by incubating with 50 µl of filtered BCIP/NBT Liquid Substrate System (SigmaAldrich) for 10-30 minutes at room temperature in darkness. The formation of IFN-γ colored spots was monitored in an ELISPOT reader (Cellular Technology Ltd., C.T.L.) after washing the plate with water and letting it get dry for 10 minutes at 37ºC.

## 11. Measuring cellular conjugates

Cellular conjugates comprising uninfected or HIV-1–infected MOLT cells and primary target cells were quantified as previously described [191]. To analyze the formation of conjugates between mDC and autologous or allogeneic non-activated primary $CD4^+$ T cells, we proceeded as follows. Purified $CD4^+$ T cells were labeled with

15 µM of CellTracker™ Orange CMRA fluorescent probe (Molecular Probes, Invitrogen) for 20 minutes at 37ºC, washed twice with PBS and left overnight at 37°C in 5% $CO_2$ in RPMI-1640 containing 10% FBS. Before performing the assays, labeled cells were washed again and resuspended in RPMI-1640 with 10 % FBS. For those experiments performed in the presence of HIV-1, mDC were incubated with $HIV_{NL4-3}$, $HIV_{NFN-SX}$ or $HIV_{NL4-3\Delta ENV}$ (50 ng $p24^{Gag}$ per $1.5x10^5$ mDC), obtained from transfection of HEK-293T cells, for 4 hours at 37ºC in 5%$CO_2$. To remove uncaptured viral particles, mDC were extensively washed with PBS. For blocking assays, mDC and CMRA-labeled $CD4^+$ T cells were separately pre-incubated for 30 minutes at room temperature in the presence or absence of the same mAb and reagents used to evaluate the adhesion molecules in viral transmission across the infectious synapse (Table 5). Then, 75,000 mDC were co-cultured with 75,000 autologous or allogeneic CMRA-labeled $CD4^+$ T cells for different incubation periods depending on the experiment (0 minutes, 30 minutes, 1 hours, 2 hours or 24 h) at 37°C in 5% $CO_2$, in a final volume of 200 µl of RPMI-1640 containing 10% FBS on a 96 well flat-bottom plate, with and without shaking. Afterwards, 50 µl of formaldehyde 2% was added to the culture without perturbing cellular conjugates and samples were analyzed in an LSR II flow cytometer equipped with a plate loader (BD Bioscience). All events with similar morphology to mDC (SSC) but simultaneously positive for the cell tracker CMRA were considered as stable cellular conjugates of mDC and primary $CD4^+$ T cells. Gating strategy for quantification of mDC–$CD4^+$ T-cell conjugates is shown in Fig. 22 A. The percentage of cellular conjugates was calculated as follows: [conjugates/total CD4 $CMRA^+$ cells]*100. Controls consisting of CMRA-labeled $CD4^+$ T cells cultured alone were performed in each experiment to quantify the background levels of T-cell–T-cell conjugates, which was less than 0.01% (Fig. 22 B). Control co-cultures between DDAO-labeled mDC (CellTrace™ Far Red DDAO-SE, Molecular Proves, Invitrogen) and CMRA-labeled $CD4^+$ T cells were performed to assess that the SSC-CMRA gating strategy unequivocally quantified mDC–$CD4^+$ T-cell conjugates (Fig. 22 C). Similar percentages of mDC–$CD4^+$ T-cell conjugates were obtained in both SSC-CMRA and DDAO-CMRA dot plot analyses.

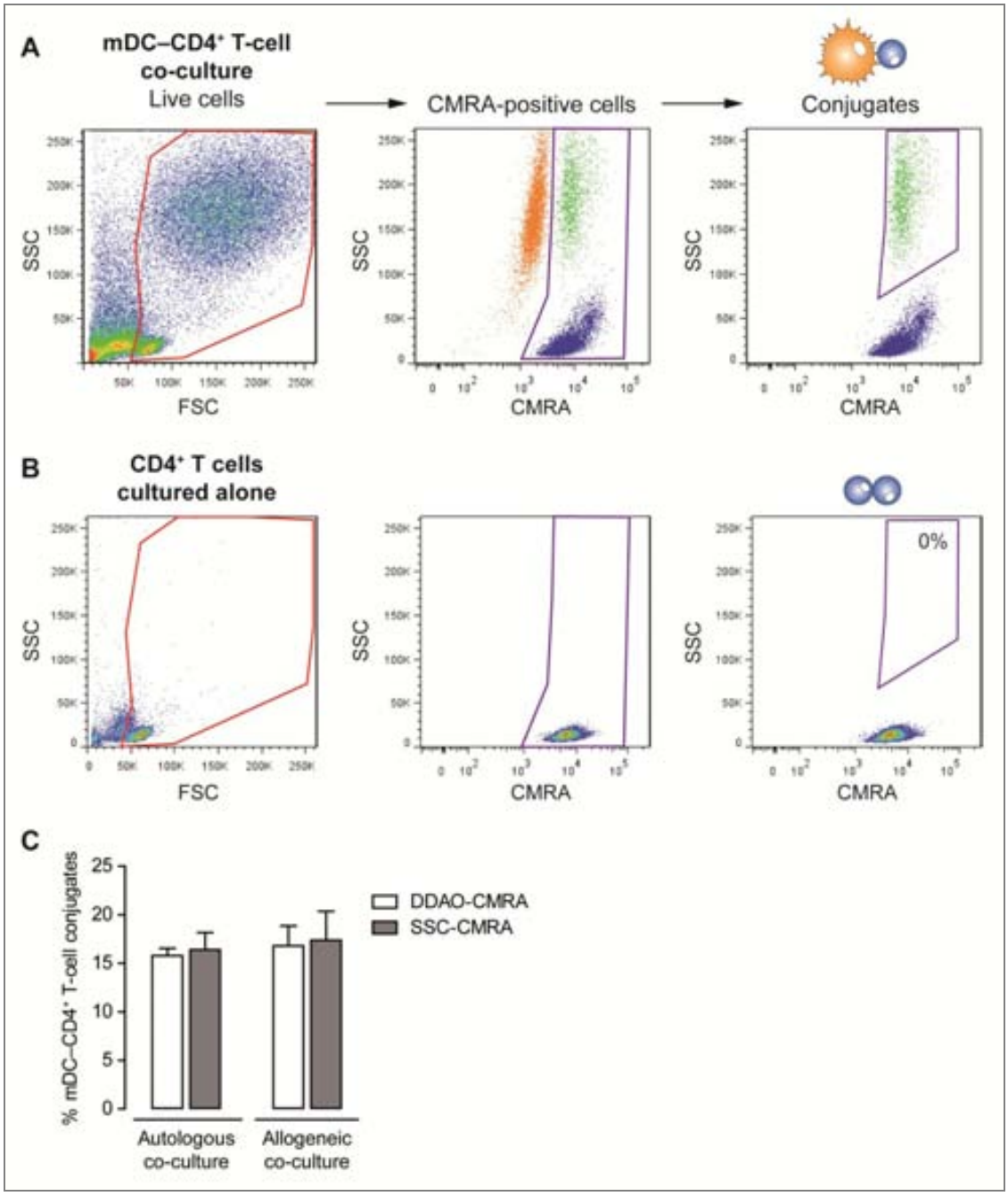


**Figure 22**. **(A)** Gating strategy for quantification of mDC–$CD4^+$ T-cell conjugates by flow cytometry. $CD4^+$ T cells were CMRA-labeled and mDC were defined based on their morphology. Live cells were first gated in FSC-SSC dot plots to discard cell debris. Then, cellular conjugates were identified within this gate and quantified in a gate including CMRA positive events. Conjugates were those events with similar morphology to mDC (SSC) but simultaneously positive for the cell tracker CMRA coming from $CD4^+$ T cells. Events corresponding to mDC are shown in orange, CMRA-labeled $CD4^+$ T cells are shown in blue, and cellular conjugates between mDC and $CD4^+$ T cells are in green. **(B)** Quantification of background levels of T-cell–T-cell conjugates. **(C)** To confirm that the gating strategy shown in panel A (SSC-CMRA) unequivocally quantified mDC–$CD4^+$ T-cell conjugates, control co-cultures between DDAO-labeled mDC and CMRA-labeled $CD4^+$ T cells were performed. Co-cultures were analyzed by the gating strategy SSC-CMRA or considering conjugates as those events simultaneously positive for the cell tackers DDAO coming from mDC and CMRA coming from $CD4^+$ T cells (DDAO-CMRA). Both quantification analyses yielded similar results.

## 12. mDC-mediated activation and proliferation of primary $CD4^+$ T cells

Activation and proliferation of non-activated primary $CD4^+$ T cells induced by mDC were analyzed after autologous or allogeneic co-culture. In brief, non-activated $CD4^+$ T cells were stained with 0.35 µM of carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Molecular Probes, Invitrogen) in PBS with 1% FBS for 6 minutes at room temperature, washed in PBS with 10% FBS and left for 30 minutes in RPMI containing 10% FBS at 37ºC in 5% $CO_2$. Cells were washed with PBS twice and finally resuspended in RPMI with 10% FBS. Then, 75,000 CFDA-SE–labeled $CD4^+$ T cells were co-cultured with 75,000 autologous or allogeneic mDC at 37°C in 5% $CO_2$ in a final volume of 250 µl of RPMI containing 10% FBS on a 96-well U-bottom plate. Previously, some mDC were incubated with $HIV_{NL4\text{-}3Ren}$ at MOI=0.1 (based on HIV-1 titration in TZM-bl cells) for 5 hours at 37ºC in 5% $CO_2$, and then cells were extensively washed with PBS to remove uncaptured viral particles. As positive controls, $CD4^+$ T cells alone or with mDC were cultured in the presence of 10 µg/ml SEA or anti-CD3 (clone OKT3) (Table 7). Negative controls based on $CD4^+$ T cells cultured alone in the absence of any stimuli were used to set the basal levels of T-cell activation which were then used to analyze the flow cytometry data of mDC–$CD4^+$ T cell co-cultures. After 16 hours or 5 days of co-culture, $CD4^+$ T-cell activation and proliferation were evaluated by staining the cells for the membrane molecules CD2, CD69, CD11c, and CD25, with the mAb listed in Table 7. Cells within the CD2-positive CD11c-negative singlet gate were analyzed. Samples were acquired on an LSR II flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star) with a built-in proliferation platform (Fig. 23).

**Table 7.** Monoclonal antibodies (mAb) and reagents used in the activation and proliferation assays between mDC and $CD4^+$ T cells.

| Molecule name | Clone | Fluorochrome | Source |
|---|---|---|---|
| CD2 | RPA-2.10 | PerCP-Cy5.5 | BD Pharmingen |
| CD3 | OKT3 | - | eBioscience |
| CD11c | Bu15 | APC-Cy7 | BioLegend |

| | | | |
|---|---|---|---|
| CD25 | M-A251 | V450 | BD Horizon |
| CD69 | FN50 | APC | BD Pharmingen |
| Staphylococcal enterotoxin A from *Staphylococcus aureus* (SEA) | - | - | SigmaAldrich |

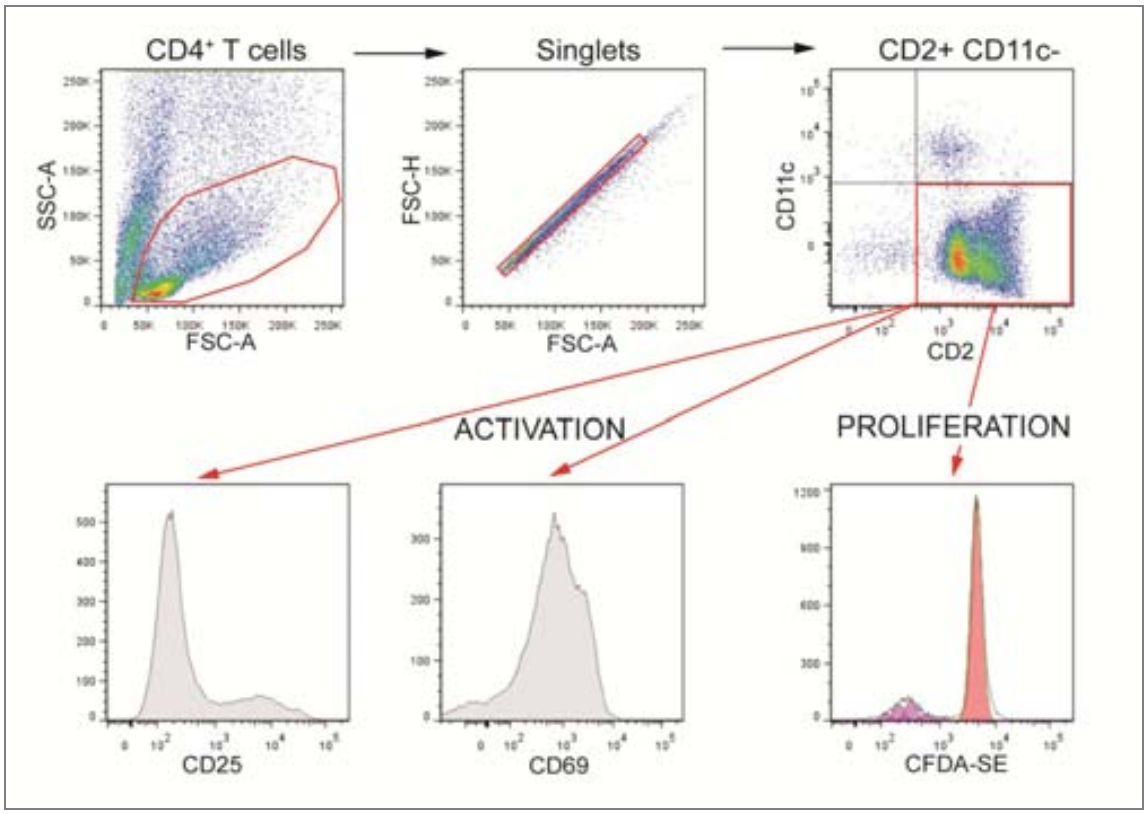


**Figure 23.** Strategy of gating to assess by flow cytometry the T cell activation and proliferation resulting from the conjugates between mDC and autologous or allogeneic primary $CD4^+$ T cells.

## 13. Microscopy

### 13.1 Confocal Microscopy

To investigate the distribution of HIV-1 in different DC subsets, a total of 2 x $10^5$ iDC and mDC matured for 48 hours with ITIP (mDC ITIP) or LPS (mDC LPS) were incubated at 37°C for 5 hours with 120 ng $p24^{Gag}$ of $HIV_{NL4\text{-}3\text{-}Vpr\text{-}eGFP}$. Cells were stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) and, after extensive washing,

were fixed with 2% formaldehyde solution and cytospun into glass-slides at 700*g* for 5 minutes. Cells were then mounted with fluorescence mounting medium (Dako) to analyze them in a confocal microscope with hybrid detector (Leica TCS SP5 AOBS; Leica Microsystems). To obtain 3D reconstructions, confocal z stacks were collected every 0.13 µm and processed with Imaris software v.7.2.3. (Bitplane AG), employing the maximum fluorescent intensity projection for $HIV_{NL4\text{-}3\text{-}Vpr\text{-}eGFP}$ and surface modelling for DAPI-stained nucleus.

To determine the polarization of the HIV-1–containing intracellular compartment in mDC to the contact zone between mDC and $CD4^+$ T cells, mDC were previously pulsed with $HIV_{NL4\text{-}3Cherry}$ for 4 hours, extensively washed with PBS, and co-cultured with $CD4^+$ T cells for 2 hours. To assess the phenotype of mDC–$CD4^+$ T cell contact, mDC and non-activated primary $CD4^+$ T cells were co-cultured for 2 hours at 37ºC in 5 $CO_2$. Then, co-cultures were stained for 30 minutes at 4ºC with α-HLA-DR and α-CD3-PE (Table 8) to unequivocally identify mDC and $CD4^+$ T cells, respectively, and washed with PBS Cells were washed with PBS. Finally, co-cultures were fixed with in 2% formaldehyde solution, cytospun onto glass slides and mounted with Fluoroshield with DAPI mounting medium (Sigma-Aldrich). Spinning disk confocal microscopy was performed on a PerkinElmer Ultraview ERS. Confocal Z-stacks were acquired at 0.25 µm steps using a 63X objective, and processed with Volocity 6.1 software (Improvision, PerkinElmer) using the maximum fluorescent intensity projection.

**Table 8.** Monoclonal antibodies (mAb) and reagents used in the activation and proliferation assays between mDC and $CD4^+$ T cells.

| Molecule name | Clone | Fluorochrome | Source |
|---|---|---|---|
| HLA-DR | L243 | Alexa Fluor 647 | BioLegend |
| CD3 | HIT3a | PE | BioLegend |

### 13.2 Electron Microscopy

For electron microscopy analysis of viral capture by DC, cells were processed as described elsewhere [169]. In brief, 3 x $10^6$ mDC LPS were pulsed at 37ºC overnight with 1,600 ng $p24^{Gag}$ of $HIV_{NL4\text{-}3}$ or $HIV_{NL4\text{-}3\Delta IN}$. Cells were then extensively washed with

PBS and fixed in 2.5% glutaraldehyde for 1 hours. Finally, cells were processed for analysis of ultrathin sections using a Jeol JEM 1010 electron microscope.

## 14. Statistical analysis

All statistical analyses were performed using GraphPad software Prism v.5. Statistical analysis was performed using non-parametric Wilcoxon matched-pairs test, parametric paired *t* test, or Spearman correlation. *P* values < 0.05 were considered statistically significant.

# Chapter 4

## RESULTS I

*HIV-1 capture and antigen presentation by dendritic cells: enhanced viral capture does not correlate with better T-cell activation*

*This chapter corresponds to the manuscript:*

**Rodriguez-Plata M.T.**, Urrutia A., Cardinaud S., Buzón M.J., Izquierdo-Useros N., Prado J.G., Puertas M.C., Erkizia I., Coulon P-G., Cedeño S., Clotet B., Moris A., Martinez-Picado J. **HIV-1 capture and antigen presentation by dendritic cells: enhanced viral capture does not correlate with better T-cell activation.** *The Journal of Immunology*. 2012 Jun 15;188(12)6036-6045.

DC are crucial in the generation and regulation of immune responses, but they may play a dual role in HIV-1 infection, by increasing the spread of HIV-1 while trying to trigger an adaptive response against viral infection [140]. Although it is well documented that the higher HIV-1 capture of mDC LPS results in increased *trans*-infection to target cells [144, 169, 186], little is known about the antigen presentation ability of this DC subset. It has recently been suggested that *Mycobacterium tuberculosis* promotes HIV-1 *trans*-infection similarly to LPS, while suppressing class II antigen processing by DC [247]. We examined whether increased HIV-1 capture in DC matured with LPS results in more efficient antigen presentation to HIV-1–specific $CD4^+$ and $CD8^+$ T cells. Using a more clinical approach, we extended the question to the proinflammatory cocktail composed of IL-1β, TNF-α, IL-6, and PGE2 (ITIP), the gold standard for DC maturation in immunotherapy [184, 248]. Moreover, in order to block the DC-mediated *trans*-infection of HIV-1, we also evaluated a non-infectious integrase-deficient HIV-1 isolate, $HIV_{NL4\text{-}3\Delta IN}$.

We showed that higher viral capture of DC did not guarantee better antigen presentation or T-cell activation. Although mDC LPS captured greater amounts of HIV-1 particles, this resulted in higher viral transmission to target cells but poor stimulation of HIV-1–specific $CD4^+$ and $CD8^+$ T cells. These data suggest that, in mDC LPS, HIV-1 exploits a specific route to be transmitted to $CD4^+$ T cells, thus significantly limiting viral antigen degradation. Conversely, maturation of DC with LPS during, but not before, viral loading enhanced both HLA-I and HLA-II HIV-1–derived antigen presentation. In contrast, DC maturation with the clinical-grade mixture ITIP during viral uptake only stimulated HIV-1–specific $CD8^+$ T cells. Hence, DC maturation state, activation stimulus, and time lag between DC maturation and antigen loading impact HIV-1 capture and virus antigen presentation. Our results demonstrate dissociation between the capacity to capture HIV-1 and to present viral antigens. Finally, as compared to replication-competent $HIV_{NL4\text{-}3}$, integrase-deficient $HIV_{NL4\text{-}3\Delta IN}$ was equally captured and presented by DC through the HLA-I and HLA-II pathways but in the absence of viral dissemination. Therefore, $HIV_{NL4\text{-}3\Delta IN}$ seems to be a promising candidate for anti-HIV-1 vaccine development. These results provide new insights into DC biology and have implications in the optimization of DC-based immunotherapy against HIV-1 infection.

## 1. Maturation of DC with LPS but not with ITIP enhances HIV-1 capture and *trans*-infection

We previously showed that mDC LPS capture greater amounts of HIV-1 than iDC, thus facilitating viral transmission to T lymphocytes [169]. Before evaluating the antigen presentation abilities of iDC and mDC, we studied the viral capture and *trans*-infection capabilities of DC matured for 48 hours with LPS or with ITIP, the gold standard for DC maturation in immunotherapy [184, 248]. In addition, we assessed the replication-competent $HIV_{NL4-3}$ and an integrase-deficient HIV-1 isolate, $HIV_{NL4-3\Delta IN}$, which allowed us to exploit viral uptake of DC in the absence of *trans*-infection.

iDC and mDC (ie, matured with ITIP [mDC ITIP] or LPS [mDC LPS] for 48 hours) were incubated with $HIV_{NL4-3}$ or $HIV_{NL4-3\Delta IN}$ for 6 hours (Fig. 24 A). After extensive washing, we lysed some cells to determine the amounts of DC-associated HIV-1. The remaining washed cells were co-cultured with TZM-bl to assay DC-mediated *trans*-infection. As expected, maturation with LPS (mDC LPS) enhanced HIV-1 capture compared with iDC (>5-fold, $p$=0.0039) (Fig. 24 B), resulting in higher *trans*-infection of $HIV_{NL4-3}$ to target cells (>7-fold; $p$=0.0156) (Fig. 24 C). Unexpectedly, maturation with ITIP (mDC ITIP) increased neither uptake nor transmission of $HIV_{NL4-3}$ and remained at similar levels to iDC (Fig. 24), even though phenotypic markers of maturation and differentiation did not diverge between mDC LPS and mDC ITIP (Fig. 25 A). To further address these differences in viral capture, HIV-$1_{NL4-3}$-Vpr-eGFP–pulsed iDC, mDC ITIP and mDC LPS were monitored by confocal microscopy (Fig. 25 B). Confirming our results by $p24^{Gag}$ ELISA (Fig. 24 B), mDC LPS captured higher amounts of viral particles, concentrating them in a large sac-like compartment, as previously described [172, 197]. On the contrary, mDC ITIP showed a random distribution of captured HIV-1, more similar to iDC than to mDC LPS.

Since vaccine adjuvants boost immune responses, mainly because they are DC activators [249, 250], we wondered whether timing of maturation during antigen loading affected viral capture and transmission to HIV-1–susceptible cells. Thus, we compared the viral uptake and *trans*-infection abilities of DC matured with LPS (iDC+LPS) and with ITIP (iDC+ITIP) during viral capture with those DC fully matured before HIV-1 incubation. Surprisingly, both iDC+ITIP and iDC+LPS exhibited a lower capacity to capture and transfer HIV-1, which was similar to iDC (Fig. 24). Therefore, only DC fully matured with LPS (mDC LPS) retained the greatest capacity to capture and transmit

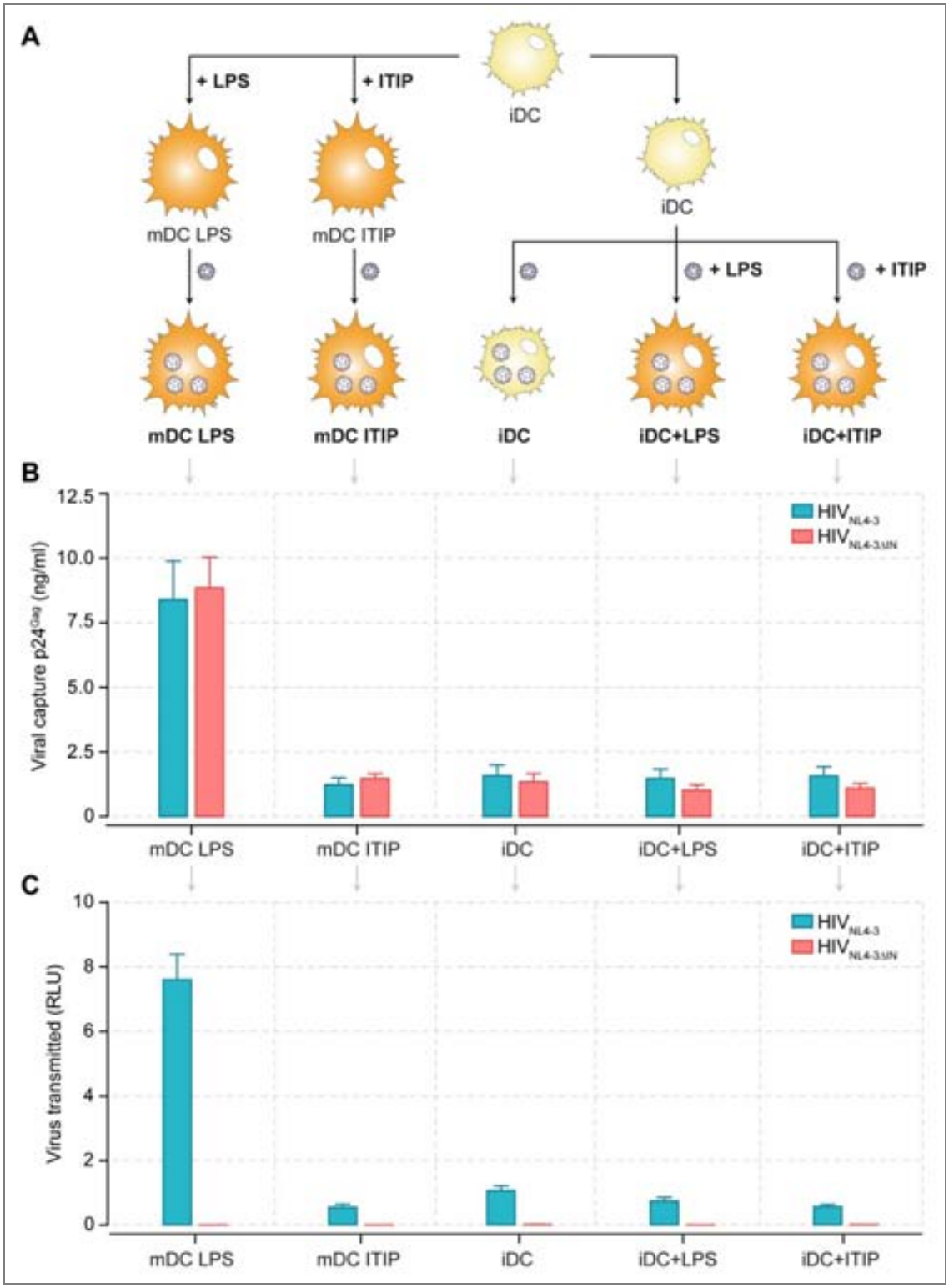


**Figure 24. Complete maturation of DC with LPS, but not with ITIP, enhances HIV-1 capture and *trans*-infection. (A)** Protocol for HIV-1 capture assay of fully mature DC matured with LPS (mDC LPS) or ITIP (mDC ITIP) for 48 hours before viral incubation, iDC, and iDC that were matured with LPS (iDC+LPS) or with ITIP (iDC+ITIP) during viral exposure. **(B)** Comparative capture of $HIV_{NL4-3}$ and $HIV_{NL4-3\Delta IN}$ under each cell condition described in panel A. The amount of DC-associated HIV-1 was determined using $p24^{Gag}$ ELISA after viral incubation at 37°C for 6 hours. Complete maturation of DC with LPS (mDC LPS), but not with ITIP (mDC ITIP), enhanced HIV-1 capture ($p$=0.0390, Wilcoxon matched-pairs test). $HIV_{NL4-3\Delta IN}$ was

captured with the same efficiency as was the wild-type $HIV_{NL4-3}$ by DC (*p*=NS, Wilcoxon matched-pairs test). Conversely, maturation of DC with LPS during viral pulse (iDC+LPS) did not enhance HIV-1 capture to levels observed in mDC LPS. **(C)** Transmission of $HIV_{NL4-3}$ and $HIV_{NL4-3\Delta IN}$ captured under each cell condition described in panel A to the TZM-bl reporter cell line. Luciferase activity was assayed after 48 hours of coculture. Background values based on non–HIV-1–exposed DC–TZM-bl cocultures were subtracted for each cell condition. In all cell subsets, viral capture correlates with viral *trans*-infection of $HIV_{NL4-3}$ to target cells, with higher ability of mDC LPS (*p*=0.0156, Wilcoxon matched-pairs test). DC-mediated *trans*-infection of $HIV_{NL4-3\Delta IN}$ was completely abrogated under all cell conditions (*p*=0.0156, Wilcoxon matched-pairs test). Data are expressed as mean and SEM and are from three independent experiments including cells from at least seven different donors.



HIV-1 to susceptible cells. Taken together, these results show that DC-mediated HIV-1 capture and transmission not only depends on DC maturation state [169], but also on the activation stimulus used for maturation [186], as well as on the time lag between DC maturation and antigen loading.

Figure 24 B, shows that $HIV_{NL4-3\Delta IN}$ was captured by DC as efficiently as replicative-competent $HIV_{NL4-3}$. As expected, functional *trans*-infection of $HIV_{NL4-3\Delta IN}$ was completely abrogated, even for mDC LPS (Fig. 24, C). Since infection of target cells was not detected, we checked whether the $HIV_{NL4-3\Delta IN}$ preserved its envelope integrity and functionality, despite lacking the whole integrase coding region. Using viral fusion assays [234], we confirmed that the $HIV_{NL4-3\Delta IN}$ was as fusogenic as the wild-type $HIV_{NL4-3}$ and equally susceptible to the C34 fusion inhibitor (Fig. 26 A). Additionally, to evaluate whether $HIV_{NL4-3\Delta IN}$ followed the same intracellular trafficking as the wild-type $HIV_{NL4-3}$ in DC, both viral particles were monitored in parallel in mDC LPS using electron microscopy (Fig. 26 B). The $HIV_{NL4-3}$ and $HIV_{NL4-3\Delta IN}$ virions had an identical structure, with a characteristic electron-dense core and similar accumulation in intracellular compartments in DC. Altogether, these findings indicated that the lack of integrase in $HIV_{NL4-3\Delta IN}$ did not alter viral fusogenicity or morphology and that $HIV_{NL4-3\Delta IN}$ behaved as a wild-type virus despite not being infectious. Thus, $HIV_{NL4-3\Delta IN}$ seems to be an attractive vaccine candidate to be explored.

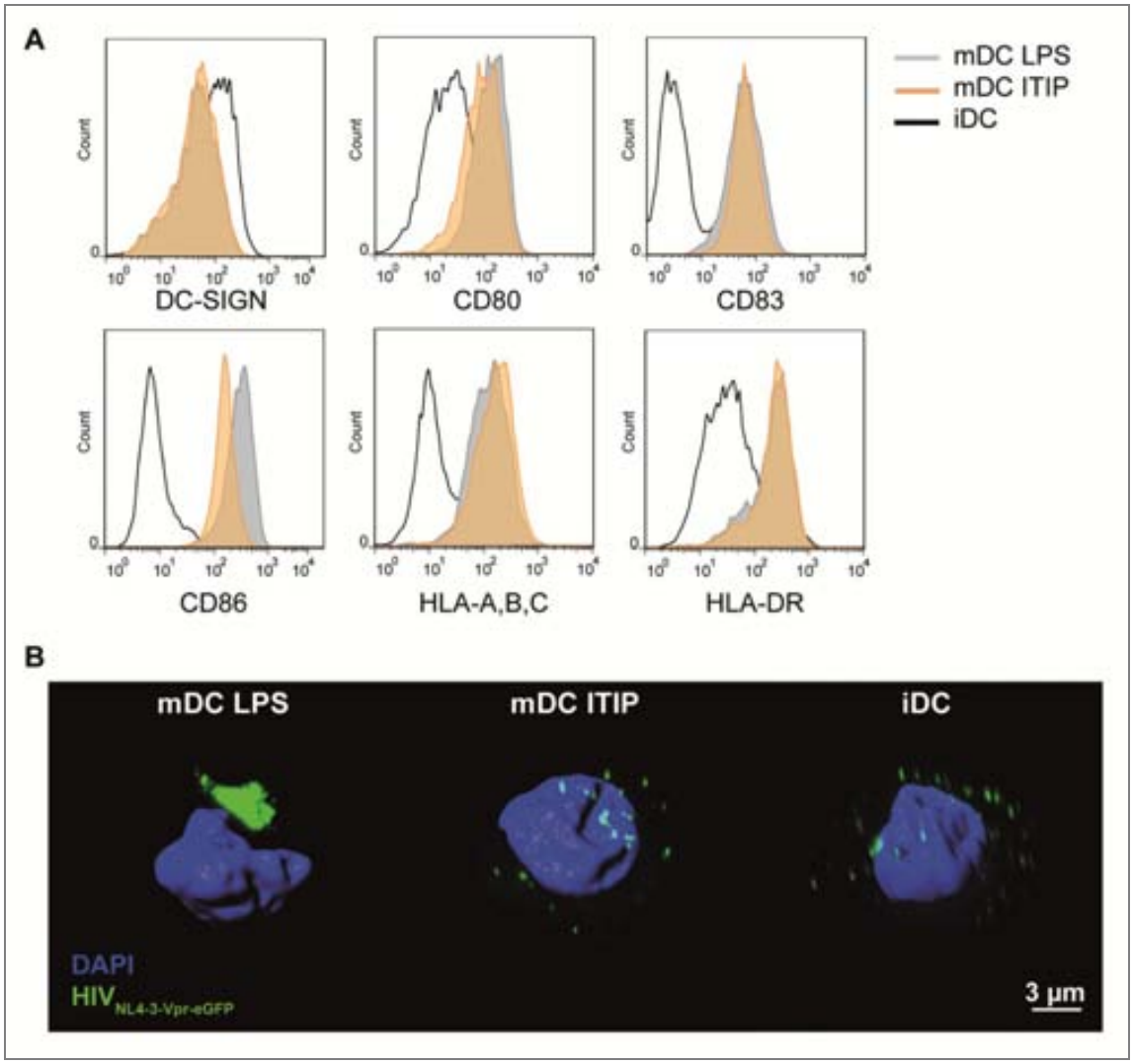


**Figure 25. mDC LPS and mDC ITIP display similar phenotypic profile but different HIV-1 localization. (A)** Immunophenotyping of iDC and mDC matured for 48 hours with ITIP (mDC ITIP) or with LPS (mDC LPS). Both maturation stimuli conferred a mature phenotype to DC, by upregulating costimulatory molecules (CD80, CD83, CD86) and HLA class-I and class-II molecules (HLA-A, -B, -C, and -DR) at the cell surface. **(B)** Confocal microscopy images of iDC and mDC matured for 48 hours with ITIP (mDC ITIP) or with LPS (mDC LPS) and exposed to $HIV_{NL4\text{-}3\text{-}Vpr\text{-}eGFP}$ for 5 hours. For three-dimensional reconstructions, confocal z stacks were collected every 0.13 µm and processed with Imaris software, using the maximum fluorescent intensity projection for $HIV_{NL4\text{-}3\text{-}Vpr\text{-}eGFP}$ and the surface modeling for DAPI-stained nucleus. mDC LPS captured higher amounts of viral particles, concentrating them into a large sac-like compartment, whereas mDC ITIP showed a random distribution of captured HIV-1, similar to that observed in iDC. Scale bar, 3 µm.

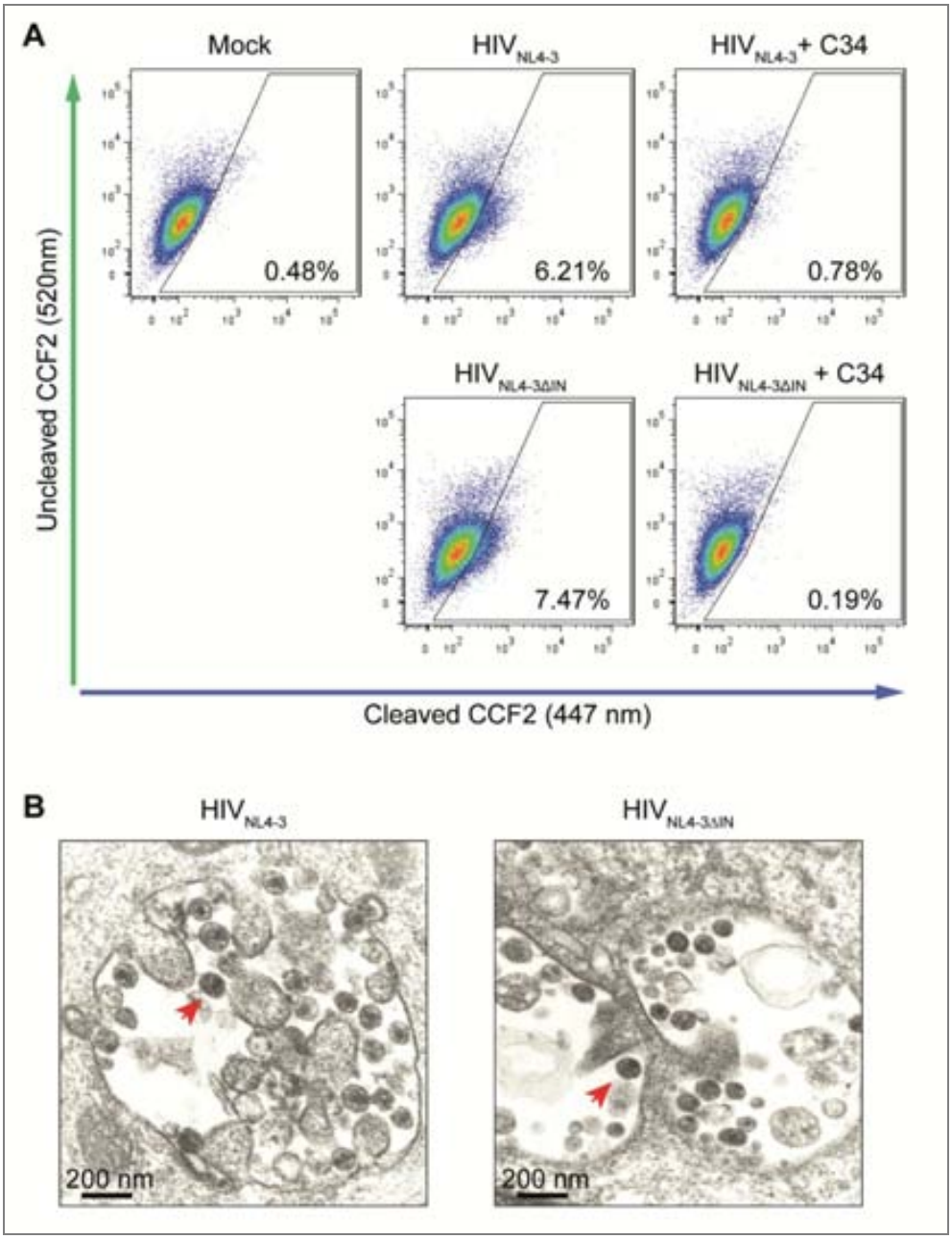


**Figure 26. The integrase-deficient HIV$_{NL4-3\Delta IN}$ is as fusogenic as the wild-type HIV$_{NL4-3}$. (A)** Viral fusion assay of HIV$_{NL4-3}$ and HIV$_{NL4-3\Delta IN}$ in the Jurkat T-cell line using HIV-1 virions containing a b-lactamase-Vpr chimeric protein. Experiments were performed in the presence or absence of C34 fusion inhibitor. HIV$_{NL4-3\Delta IN}$ was as fusogenic as wild-type HIV$_{NL4-3}$ and equally susceptible to the C34 fusion inhibitor. **(B)** Comparative electron microscopy images of mDC LPS exposed to HIV$_{NL4-3}$ (left panel) or HIV$_{NL4-3\Delta IN}$ (right panel), showing similar large vesicle location. Red arrows indicate captured particles, which have the characteristic electron-dense structure of the HIV-1 core. Processing and analysis using a Jeol JEM 1010 electron microscope.

## 2. Enhanced viral capture does not correlate with better T-cell activation

In order to elucidate whether viral capture correlates with antigen presentation and T-cell activation, we evaluated the abilities of iDC, mDC ITIP, mDC LPS, iDC+ITIP, and iDC+LPS to present HIV-1–derived antigens to $CD4^+$ and $CD8^+$ T cells.

To this end, we used various previously generated HIV-1–specific T-cell clones [104, 165]. To monitor HLA-I HIV-1 antigen presentation, we used two different $CD8^+$ T-cell clones, SL9-2 and EM40-F21, which are specific for HIV-1 $p17^{Gag}$ (aa 77-85), restricted by HLA-A*02, and derived from two HIV-1–infected patients [104]. As the wild–type $HIV_{NL4-3}$ and its derived $HIV_{NL4-3\Delta IN}$ did not present the consensus SL9 epitope restricted by HLA-A*02, we engineered $HIV_{NL4-3}$ and $HIV_{NL4-3\Delta IN}$ to express the optimal SL9 sequence (SLYNTVATL) [242] or the escape variant (SLFNTIAVL) [243] of the SL9 epitope. Two $CD4^+$ T-cell clones, F12 and N2, which are specific for HIV-1 $p24^{Gag}$ (aa 271-290) and restricted by HLA-DRβ*04/HLA-DRβ*01 and HLA-DRβ*04, respectively, were used to evaluate HLA-II antigen presentation [165]. HLA-matched DC for each HIV-1–specific T-cell clone were exposed for 24 hours (HLA-I assays) or 6 hours (HLA-II assays) to $HIV_{NL4-3}$ or $HIV_{NL4-3\Delta IN}$ (Fig. 27). T-cell activation was monitored using IFN-γ ELISPOT after overnight co-culture of HIV-1–pulsed DC with HIV-1–specific T-cell clones. Since it is known that after activation with LPS, DC transiently produces IL-12 becoming subsequently refractory to further stimulation [251], and that DC activation with PGE2 suppresses IL-12 production [252], we performed several negative controls consisting of non-HIV-1–exposed DC–T-cell clone co-cultures or SL9 escape HIV-1 variants in the ELISPOT assays, to discard the non-specific activation of the T-cell clones and eliminate potential background due to IL-12 secretions. Figure 27 shows a representative experiment using SL9-2 and F12 clones for HLA-I and HLA-II presentation, respectively. All assays were performed in the presence of NVP and AZT to prevent viral replication and guarantee that activation of HIV-1–specific $CD8^+$ and $CD4^+$ T-cell clones was not due to the presentation of *de novo* viral proteins synthesized in DC.

Remarkably, compared with iDC, mDC LPS loaded with $HIV_{NL4-3}$ or $HIV_{NL4-3\Delta IN}$ did not enhance HIV-1–specific $CD8^+$ T-cell activation (Fig. 27 B). This contrasts with the extremely high viral capture and HLA molecule expression observed in mDC LPS (Fig. 24, Fig. 28 A and C, Fig. 29 A and C). Furthermore, inducing full DC maturation with

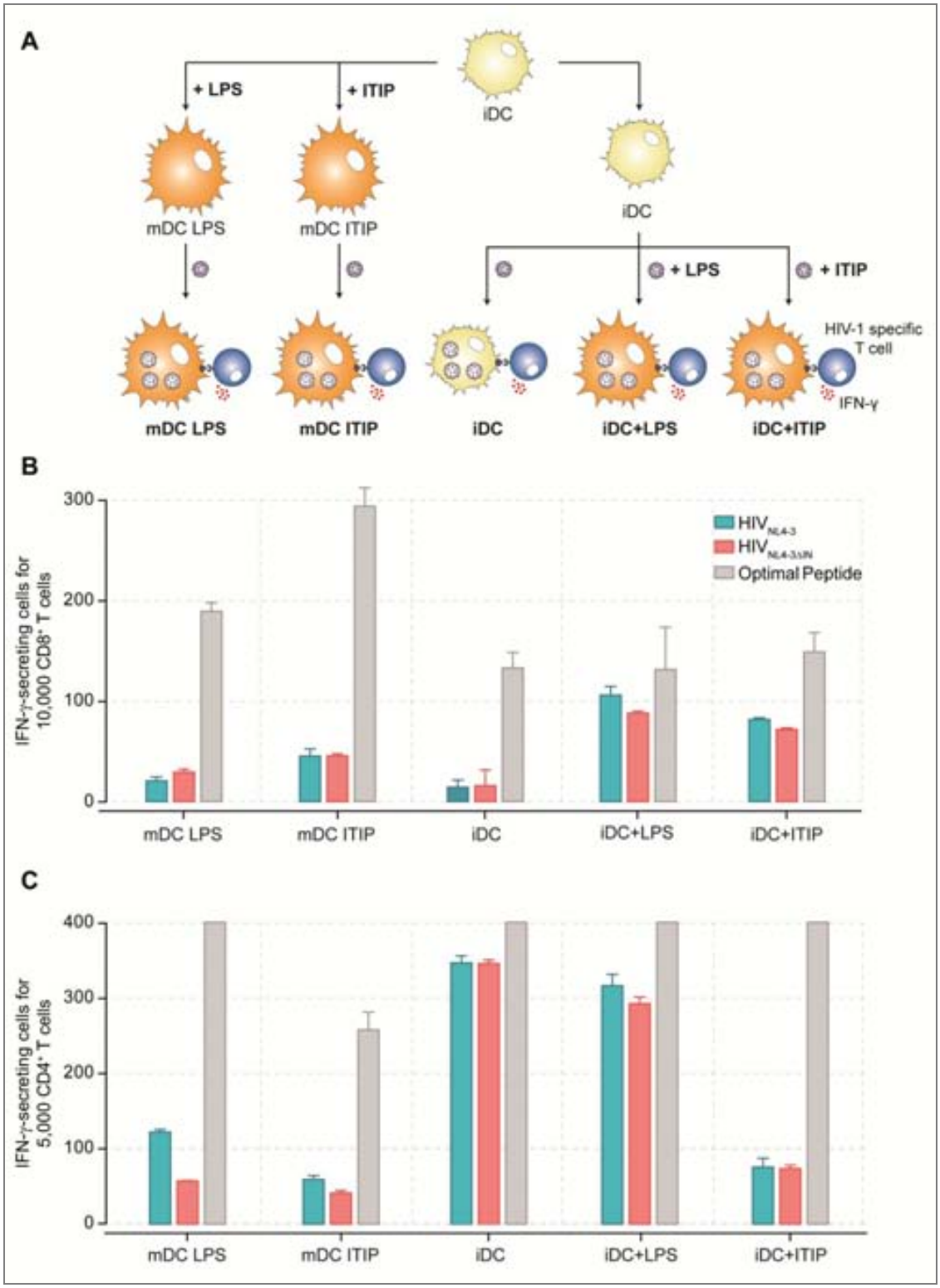


**Figure 27. Increased viral capture does not correlate with better T-cell activation. DC maturation with LPS during viral capture enhanced both HLA-I and HLA-II antigen presentation. (A)** Protocol for HLA-I and HLA-II antigen presentation assays of iDC that were matured with ITIP (iDC+ITIP) or with LPS (iDC+LPS) during viral incubation, as well as fully mature DC that were matured with ITIP (mDC ITIP) or with LPS (mDC LPS) for 48 hours before viral incubation. The SL9-2 and EM-40-F21 HIV-1 $p17^{Gag}$ SL9 $CD8^+$ T-cell clones (aa 77–85), restricted by HLA-A2, were used for the analysis of exogenous HLA-I antigen presentation, and the F12 and N2 HIV-1 $p24^{Gag}$ $CD4^+$ T-cell clones (aa 271–290), restricted by HLA-DRβ*01/HLA-

DRβ*04 and HLADRβ* 04, respectively, were used to evaluate HLA-II antigen presentation. HLA-matched DC for each HIV-1–specific T-cell clone were exposed for 24 hours (HLAI assays) or 6 hours (HLA-II assays) to $HIV_{NL4-3}$ or $HIV_{NL4-3\Delta IN}$. Antigen presentation was quantified using IFN-γ ELISPOT after overnight coculture of HIV-1–pulsed DC with HIV-1–specific T-cell clones. All assays were performed in the presence of NVP and AZT to prevent viral replication. **(B)** The high viral capture of mDC LPS (see Fig. 24 B) did not result in greater HIV-1–specific $CD8^+$ activation when loaded with $HIV_{NL4-3}$ or $HIV_{NL4-3\Delta IN}$. Nevertheless, maturation with ITIP and LPS during HIV-1 loading (iDC+ITIP and iDC+LPS, respectively) induced greater activation of the SL9-2 HIV-1–specific $CD8^+$ T cells. Both $HIV_{NL4-3}$ and $HIV_{NL4-3\Delta IN}$ were equally cross-presented to HIV-1–specific $CD8^+$ T cells. As a positive control, DC were incubated with 0.1 mg/ml of cognate peptide under the same conditions as HIV-1–pulsed cells, with optimal activation. Negative-control values consisting of non–HIV-1–exposed DC–$CD8^+$ T-cell clone cocultures were subtracted from each cell condition to specifically show the response to HIV-1–derived antigens. The panel shows one representative experiment of two (using two different HIV-1–specific $CD8^+$ T-cell clones) that yielded similar results. Experiments were performed in triplicate and at three E:T ratios. Data are expressed as mean and SEM. **(C)** iDC presented the HIV-1–derived antigens more efficiently to HIV-1–specific $CD4^+$ T cells than did fully mature DC (either mDC ITIP or mDC LPS). Despite their increased capacity for viral capture, mDC LPS loaded with $HIV_{NL4-3}$ or $HIV_{NL4-3\Delta IN}$ did not enhance F12 HIV-1–specific activation of $CD4^+$ (see Fig. 24 B). Nevertheless, LPS maturation of iDC during viral pulse (iDC+LPS) substantially increased activation of HIV-1–specific $CD4^+$ T cells. $HIV_{NL4-3\Delta IN}$ induced an HIV-1–specific $CD4^+$ T-cell response comparable to that observed for $HIV_{NL4-3}$ under all DC conditions. As a positive control, DC were incubated with 0.1 mg/ml of cognate peptide under the same conditions as HIV-1–pulsed cells, with optimal activation. Negative-control values consisting of non–HIV-1–exposed DC–$CD4^+$ T-cell clone cocultures were subtracted from each cell condition to specifically show the response to HIV-1–derived antigens. The panel shows one representative experiment of two (using two different HIV-1–specific $CD4^+$ T-cell clones) that yielded similar results. Experiments were performed in triplicate and at three E:T ratios. Data are expressed as mean and SEM.

ITIP (mDC ITIP) had a moderate effect, if any, on HIV-1–specific CTL activation (Fig. 27 B). It has been reported that HLA-I–restricted exogenous HIV-1 antigen presentation requires fusion of viral and cellular membranes in a CD4/co-receptor–dependent manner and release of $HIV\text{-}1^{Gag}$ capsid into the cytosol of DC for proteasomal processing and HLA-I loading [253, 254]. However, maturation of DC is associated with a decline in HIV-1 fusion [147], which, in turn, has a direct impact on the ability of mDC to support viral replication [144, 147]. These observations most likely explained why DC matured with either LPS or ITIP (mDC LPS and mDC ITIP, respectively) exhibiting very high levels of HLA-I and co-stimulatory molecules induced very low stimulations of HIV-1–specific $CD8^+$ T-cell clones.

Interestingly, compared with mDC ITIP and mDC LPS, iDC induced a 3-fold enhancement of HIV-1–specific $CD4^+$ T-cell clone activation (Fig. 27 C), in contrast to their reduced expression of HLA-II and co-stimulatory molecules and their poor capacity to capture HIV-1 virions (Fig. 24 B, Fig. 28 B and D. Fig. 29 B and D). These results strongly suggest that HIV-1 capture by mDC LPS does not route HIV-1 virions towards degradation compartments and HLA loading. Our results are reminiscent of

the observations that localization of captured virus differs between iDC and mDC [181], as mDC—but not iDC—accumulate whole virions in a non-conventional endocytic compartment rich in tetraspanins with a mildly acidic pH [163, 169, 172]. In addition, HLA-II antigen presentation depends on viral degradation in acidified endosomes [254]; however, inhibition of endosomal acidification preserves HIV-1 infectivity [255].



### 3. DC maturation with LPS during viral capture enhanced both HLA-I and HLA-II antigen presentation

We next examined the effect of maturation of iDC during HIV-1 loading in antigen presentation by HLA-I and HLA-II molecules. iDC were simultaneously matured with ITIP or LPS (iDC+ITIP and iDC+LPS, respectively) and pulsed with $HIV_{NL4-3}$ or $HIV_{NL4-3\Delta IN}$ for 24 hours in HLA-I antigen presentation assays or 6 hours in HLA-II experiments. After overnight co-culture of HIV-1–pulsed DC with HIV-1–specific T-cell clones, antigen presentation was quantified by IFN-γ ELISPOT (Fig. 27). All assays were performed in the presence of NVP and AZT to ensure that antigens did not derive from neo-synthesized HIV-1 proteins.

In contrast to fully matured DC (mDC LPS and mDC ITIP), DC stimulated with LPS or ITIP simultaneously with virus loading (iDC+LPS and iDC+ITIP) induced higher activation of HIV-1–specific $CD8^+$ T cells than iDC (Fig. 27 B). Minor differences were observed in HLA-I antigen presentation between DC matured with LPS and ITIP, although iDC+LPS represented a 3-fold increase in IFN-γ secretion and iDC+ITIP a 1.5-fold change compared to their fully matured counterparts. It is worth noting that HIV-1–specific $CD8^+$ T-cell activation levels induced by peptide-loaded mDC LPS and mDC ITIP were higher than levels induced by peptide-loaded iDC, iDC+LPS, and iDC+ITIP (Fig. 27 B), most likely reflecting higher HLA-I molecule expression (Fig. 28 C). In contrast, upon loading of the DC subsets with $HIV_{NL4-3}$ or $HIV_{NL4-3\Delta IN}$, neither viral capture nor HLA-I molecule expression correlated with better $CD8^+$ T-cell activation (Fig. 28 A and C, 29 A and C). As expected, neither $HIV_{NL4-3}$ nor $HIV_{NL4-3\Delta IN}$ expressing the escape variant (SLFNTIAVL) of the SL9 epitope induced responses in the SL9-2 $CD8^+$ T-cell clone under any DC condition (Fig. 30). As stated elsewhere [147], DC maturation restricts viral fusion, which is crucial for cytosolic proteasomal processing of Gag proteins and proper HLA-I antigen presentation [253, 254]. Thereafter, although

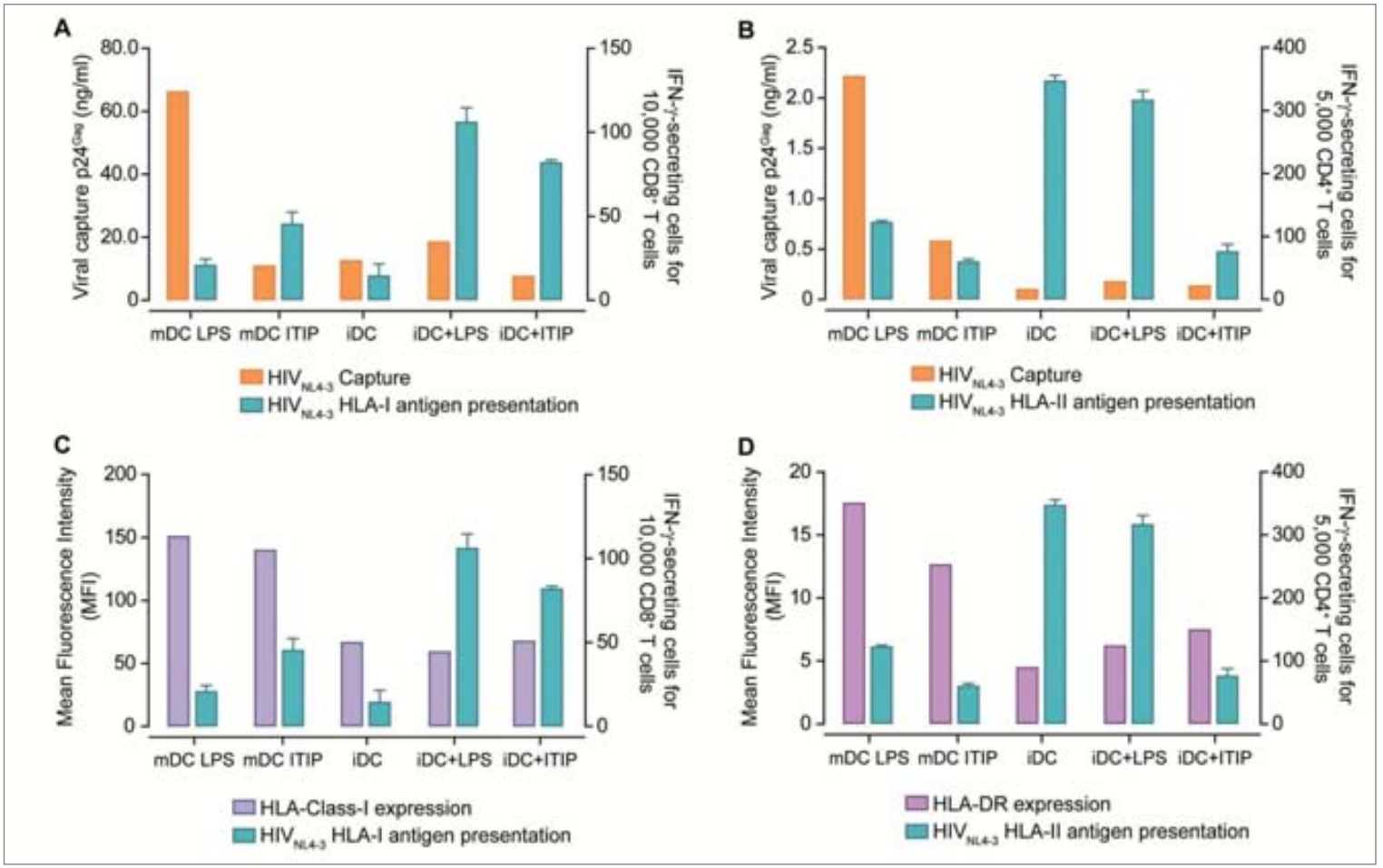


**Figure 28. Antigen presentation does not correlate with viral capture or HLA expression by DC (results for $HIV_{NL4-3}$).** Comparative analysis of IFN-γ production by the SL9-2 $CD8^+$ T cell clone **(A)** and the F12 $CD4^+$ T cell clone **(B)** in antigen-presentation assays and DC viral capture, as evaluated by measurement of intracellular $p24^{Gag}$ $HIV_{NL4-3}$ content under each DC condition tested in Fig. 27 before launching the ELISPOT assay. Relationship between IFN-γ production by SL9-2 $CD8^+$ T cell clone and HLA class-I expression **(C)** and by F12 $CD4^+$ T cell clone and HLA-DR expression **(D)**.

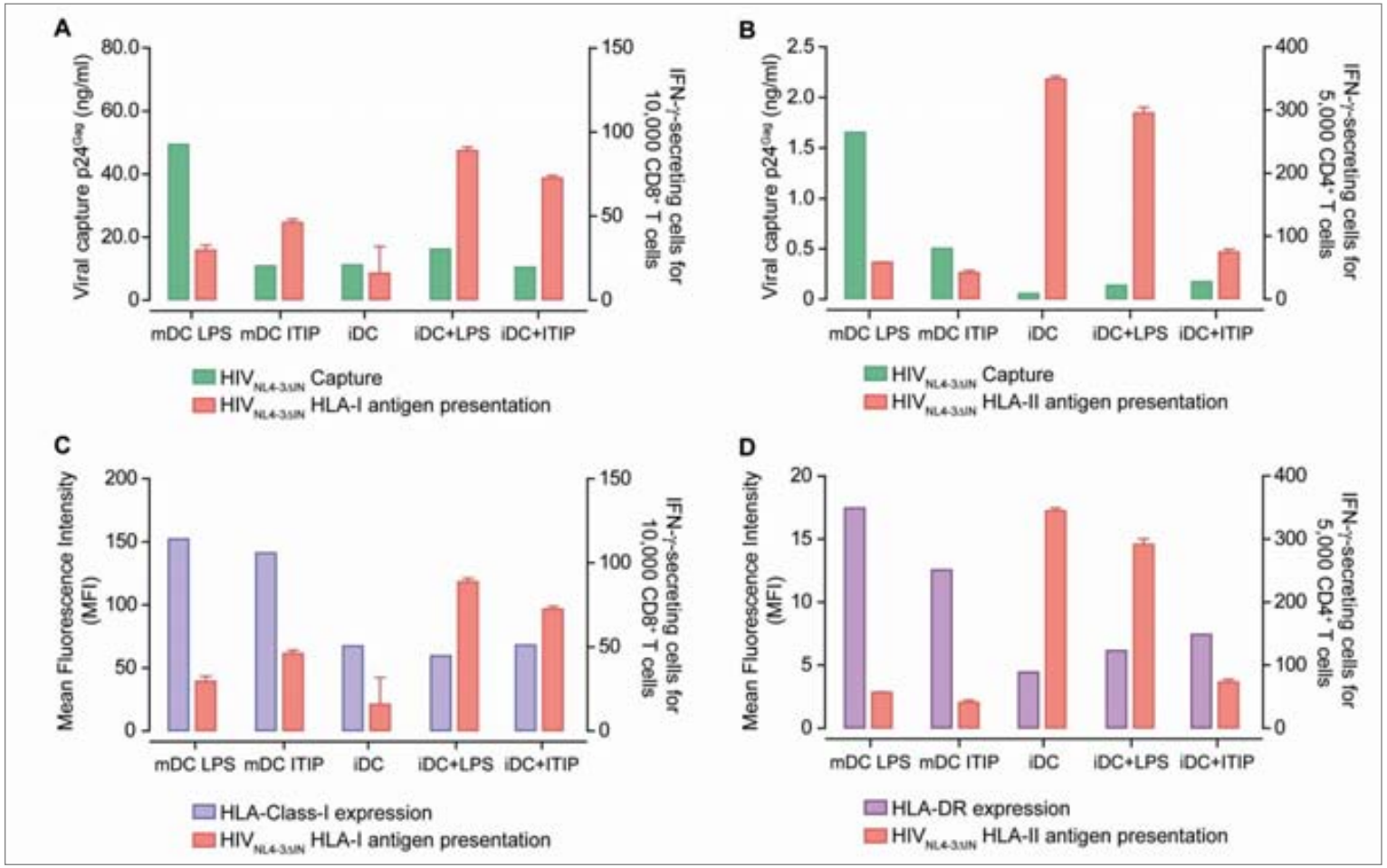


**Figure 29. Antigen presentation does not correlate with viral capture or HLA expression by DC (results for $HIV_{NL4\text{-}3\Delta IN}$).** Comparative analysis of IFN-γ production by the SL9-2 $CD8^+$ T cell clone **(A)** and the F12 $CD4^+$ T cell clone **(B)** in antigen-presentation assays and DC viral capture, as evaluated by measurement of intracellular $p24^{Gag}$ $HIV_{NL4\text{-}3}$ content under each DC condition tested in Fig. 27 before launching the ELISPOT assay. Relationship between IFN-γ production by SL9-2 $CD8^+$ T cell clone and HLA class-I expression **(C)** and by F12 $CD4^+$ T cell clone and HLA-DR expression **(D).**

iDC capture lower amounts of virions, HIV-1 is more prone to fuse, potentially enhancing viral uptake and HLA-I antigen presentation of HIV-1–derived peptides.

Compared to mDC LPS and mDC ITIP, LPS maturation of iDC simultaneously with viral pulse (iDC+LPS) increased activation of HIV-1–specific $CD4^+$ T cells, reaching levels similar to those induced by iDC (Fig. 27 C). Once again, the capacity to activate HIV-1–specific $CD4^+$ T-cell clones did not correlate with the capacity to capture HIV-1 or HLA-II expression levels (Fig. 24 B, 28 B and D, 29 B and D). On the other hand, maturation of iDC with ITIP during antigen uptake (iDC+ITIP)—rather than before antigen uptake (mDC ITIP)—did not improve HLA-II antigen presentation (Fig. 27). These results show that maturation of DC with ITIP simultaneously with antigen loading (iDC+ITIP) does not guarantee efficient $CD4^+$ T-cell activation. The type of activation (ITIP- or LPS-induced) also determines the capacity to process and present antigens.

Interestingly, DC loaded with integrase-deficient $HIV_{NL4\text{-}3\Delta IN}$ induced comparable activation of both HIV-1–specific $CD8^+$ and $CD4^+$ T-cell clones than DC loaded with $HIV_{NL4\text{-}3}$, further demonstrating that no viral replication was needed for HIV-1–derived antigen presentation. $HIV_{NL4\text{-}3\Delta IN}$ is a promising immunogen for the development of an HIV-1 vaccine, since it was processed and presented by DC as wild-type HIV-1 without being infectious.

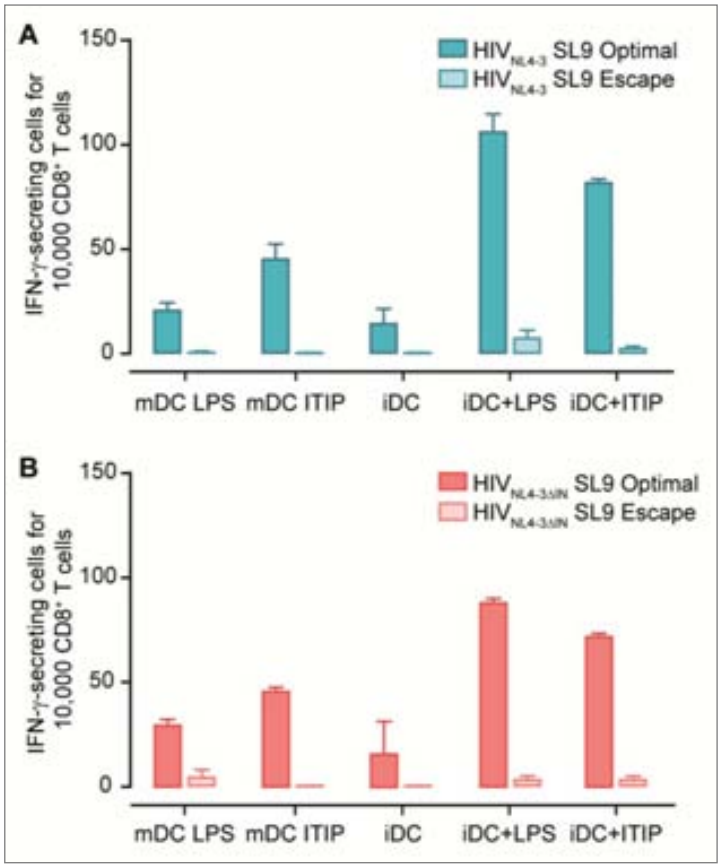


**Figure 30.** Comparative analysis of IFN-γ production by the SL9-2 $CD8^+$ T-cell clone when stimulated with DC pulsed with $HIV_{NL4\text{-}3}$ (A) or $HIV_{NL4\text{-}3\Delta IN}$ (B) expressing the optimal (SLYNTVATL) or the escape (SLFNTIAVL) variant of SL9 epitope. Both $HIV_{NL4\text{-}3}$ and $HIV_{NL4\text{-}3\Delta IN}$ expressing the escape variant for the SL9 epitope did not induce activation of the HIV-1–specific $CD8^+$ T-cell clone response.

# Chapter 5

## RESULTS II

*The infectious synapse formed between mature dendritic cells and CD4$^+$ T cells is independent of the presence of the HIV-1 envelope glycoprotein*

*This chapter corresponds to the manuscript:*

**Rodriguez-Plata M.T.**, Puigdomènech I., Izquierdo-Useros N., Puertas M.C., Carrillo J., Erkizia I., Clotet B., Blanco J., Martinez-Picado J. **The infectious synapse formed between mature dendritic cells and CD4$^+$ T cells is independent of the presence of the HIV-1 envelope glycoprotein.** *Retrovirology.* 2013 Apr 16;10(1):42.

DC play a key role in the initiation of primary T-cell–mediated immune responses *in vivo*, as they are the most potent APC in the immune system, especially upon maturation [29]. DC launch the adaptive immune responses with the interaction of the appropriate pMHC with the TcR, which constitutes the basis of the immunological synapse. This immunological synapse provides sustained T-cell signaling, leading to T-cell priming and TcR downregulation [256, 257]. But DC can also interact with T cells in the absence of cognate antigen [120-122]. The initial stages of DC–$CD4^+$ T-cell conjugate formation are actually antigen-independent, suggesting that DC–T cell adhesion precedes antigen recognition [120, 121]. Although antigen-independent interactions do not induce full activation of T cells—as occurs in antigen-dependent interactions—they do maintain the homeostasis of naïve T cells [120, 122, 123].

However, HIV-1 has evolved strategies to subvert DC antiviral activity [202, 258], given that cell-mediated infection is much more efficient than cell-free infection [179]. Consequently, HIV-1 manipulates the function of DC to enhance their spread, survival and transmission, taking advantage of the continuous interplay between DC and T lymphocytes [140]. DC can support HIV-1 dissemination through the release of new virus particles in the course of productive infection (*cis*-infection) or through the transfer of captured HIV-1 particles to target cells without DC infection (*trans*-infection) [144, 149]. Since HIV-1 replication in DC is very inefficient, *trans*-infection is considered the main mechanism of DC-mediated viral transmission [140]. *Cis*-infection of target cells occurs across the virological synapse, a cellular contact that is driven by the cell-surface expression of the viral envelope glycoprotein in the infected cell reported [176, 191, 192, 197]. On the contrary, *trans*-infection occurs via the infectious synapse, a cell-to-cell contact zone that facilitates transmission of HIV-1 by locally concentrating virus and viral receptors [163, 177]. The structure of the infectious synapse is thought to have similarities with the immunological synapse [259], but little is known about the role of viral envelope glycoprotein during contact between DC and T cells.

In this study, we explored the contribution of HIV-1 Env during conjugate formation at the infectious synapse and analyzed its role in combination with adhesion molecules in the context of antigen presentation. Our data showed that, in contrast with the virological synapse, HIV-1 did not modulate the formation of the infectious synapse between mDC LPS harboring HIV-1 and uninfected $CD4^+$ T cells. On the contrary, the main driving force behind formation of mDC–$CD4^+$ T-cell conjugates and HIV-1 *trans*-infection of $CD4^+$ T cells was the interaction between ICAM-1 and LFA-1. In addition, antigen recognition or a sustained MHC-TcR interaction did not enhance conjugate

formation, but significantly boosted productive DC-mediated HIV-1 *trans*-infection. Consistently, antigen recognition also significantly increased T-cell activation and proliferation after conjugation with mDC LPS. Our results suggest a determinant role of contact between mDC and $CD4^+$ T cells in immune activation and viral dissemination, which likely contribute to the pathogenesis of HIV-1 infection.

## 1. mDC–CD4$^+$ T-cell conjugate formation is HIV-1–independent

The interaction between the viral Env on the surface of the HIV-1–infected cell and its primary receptor, CD4, on the target cell is one of the determinants in the formation of the virological synapse, as we and other authors have reported [176, 191, 192, 197]. Moreover, the dependence of the virological synapse on Env increases the frequency of stable cell conjugates comprising HIV-1–infected and uninfected T cells, thus facilitating the transfer of virus to target cells [176, 191, 192, 197]. However, the role of HIV-1 Env in the cell-to-cell interaction at the infectious synapse between uninfected mDC harboring HIV-1 and target CD4$^+$ T lymphocytes has not yet been addressed. Since mDC LPS capture large amounts of HIV-1 particles [169, 186], and polarizes the HIV-1–containing intracellular compartment to the contact zone with target cells (Fig. 31 A) [163, 177, 183], the Env of trapped virions could guide the formation of mDC–CD4$^+$ T-cell conjugates.

In order to evaluate whether the presence of HIV-1 also drives the formation of conjugates comprising mDC harboring HIV-1 and uninfected CD4$^+$ T cells, we used a previously described flow cytometry method [191] to quantify cellular conjugates between CMRA-labeled uninfected CD4$^+$ T cells and mDC LPS pulsed or not with viral particles. This approach enabled us to identify cellular conjugates as those events with a similar morphology to mDC LPS and high levels of fluorescence from the CMRA-labeled CD4$^+$ T cells (Fig. 22 A and 31 B). In addition, we also assessed the contribution of antigen recognition during conjugate formation. To that end, we used autologous and allogeneic co-cultures of mDC LPS and non-activated CD4$^+$ T cells in the presence or absence of HIV-1 to evaluate antigen-independent and antigen-dependent interactions, respectively. Although alloreactivity does not involve an antigen-specific response, allorecognition is characterized by a stable MHC-TcR interaction that elicits an exceptionally vigorous T-cell response [260]. Besides, as many as 1-10% of T lymphocytes can respond to allogeneic MHC molecules [261, 262]; this frequency is several orders of magnitude above the frequency of specific T cells for any single foreign antigen presented by self-MHC [260, 263].

Unlike the virological synapse, the presence of HIV-1 did not increase the formation of conjugates between mDC LPS harboring HIV-1 and non-activated primary CD4$^+$ T cells. Thus, unpulsed mDC LPS or mDC LPS pulsed with X4-tropic $HIV_{NL4-3}$, R5-tropic $HIV_{NFN-SX}$, or Env-deficient $HIV_{NL4-3\Delta ENV}$ displayed the same percentage of

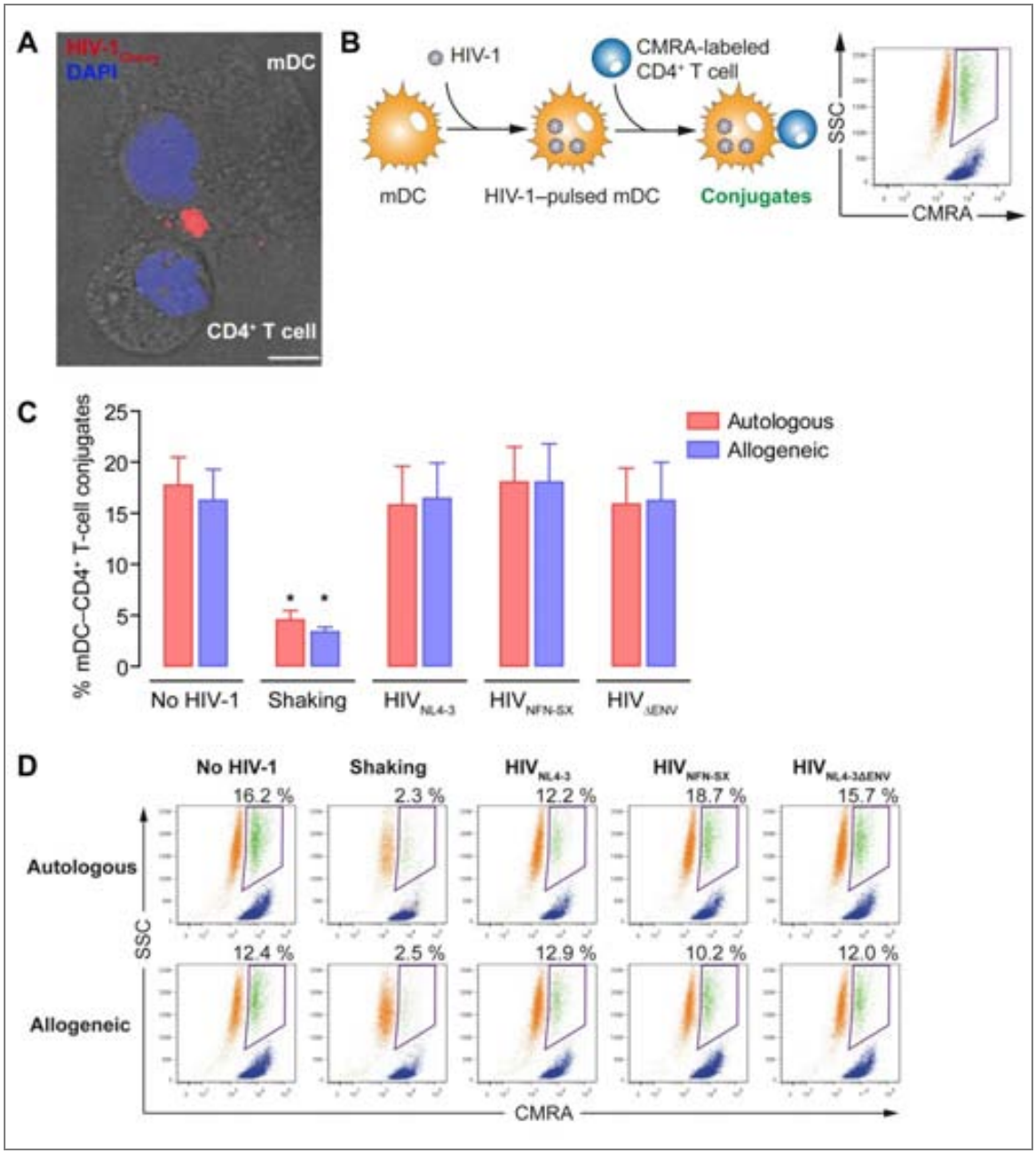


**Figure 31. Conjugate formation between mDC LPS harboring HIV-1 and primary CD4$^+$ T cells is independent of the presence of HIV-1. (A)** Confocal microscopy analysis of a HIV-1–pulsed mDC–CD4$^+$ T-cell synapse, showing the polarization of the HIV-1–containing intracellular compartment in mDC LPS to the contact zone. Merge of the bright field and the fluorescence of an x-y plane (scale bar: 5 µm). **(B)** Experimental procedure for quantification of cellular conjugates by flow cytometry. mDC LPS were incubated or not with $HIV_{NL4-3}$, $HIV_{NFN-SX}$, or $HIV_{NL4-3\Delta ENV}$, before being co-cultured with CMRA-labeled CD4$^+$ T cells for 2 hours. All events with a similar morphology to that of mDC LPS (SSC) but simultaneously positive for the cell tracker CMRA were considered stable cellular conjugates between mDC LPS and primary CD4$^+$ T cells (green). Events corresponding to mDC are shown in orange, and CMRA-labeled CD4$^+$ T cells are shown in blue. **(C)** Comparative quantification of cellular conjugates in autologous (red bars) or allogeneic (blue bars) co-cultures of mDC LPS and non-activated primary CD4$^+$ T cells in the presence or absence of HIV-1. mDC LPS were incubated or not with $HIV_{NL4-3}$, $HIV_{NFN-SX}$, or $HIV_{NL4-3\Delta env}$, before being co-cultured with non-activated CD4$^+$ T cells for 2 hours. Continuous shaking of the co-culture for 2 hours at 37ºC was used as a negative control of conjugate formation. **(D)** Representative experiment of quantification of cellular conjugates between mDC LPS preloaded with different HIV-1 strains ($HIV_{NL4-3}$, $HIV_{NFN-SX}$, and $HIV_{NL4-3\Delta ENV}$) and non-

activated CMRA-labeled $CD4^+$ T cells by flow cytometry; cellular conjugates are shown in green, mDC LPS in orange, and CMRA-labeled $CD4^+$ T cells in blue. Numbers represent the percentage of cellular conjugates in the gate. Asterisks indicate significant differences compared with co-cultures in the absence of HIV-1 ($p<0.05$, Wilcoxon matched-pairs test). Data are expressed as mean and SEM from three independent experiments including cells from six different donors.

cellular conjugates (within a range of 15.8-19.9%) with non-activated $CD4^+$ T cells (Fig. 31 C and D), revealing that viral tropism or even the HIV-1 envelope itself had no effect on the formation of mDC–$CD4^+$ T-cell conjugates. As previously described [264], limitation of cell contacts by continuous shaking significantly inhibited the formation of cellular conjugates (within a range of 3.7-5.3%) ($p<0.05$) (Fig. 31 C and D). Neither the presence of HIV-1 nor the capture and internalization of virus by mDC LPS impacted the conjugate formation with non-activated $CD4^+$ T cells.



This finding contrasts somewhat with the T cell–T cell virological synapses formed between productively infected cells and uninfected primary $CD4^+$ T cells [191, 192]. We analyzed this by using uninfected primary $CD4^+$ T cells in co-culture with the T-lymphoblastoid MOLT cell lines chronically infected with X4-tropic ($MOLT_{NL4-3}$) HIV-1 or R5-tropic ($MOLT_{BaL}$) HIV-1 and observed a higher frequency of cellular conjugates with the HIV-1–infected T cells (both 9.2%) than with uninfected MOLT cells (2.6%) (Fig. 32 A and B). In addition, the virological synapses were inhibited by blocking gp120 binding to CD4 using the α-CD4 Leu3a monoclonal antibody (mAb), reaching levels similar to those observed between uninfected MOLT cells and primary $CD4^+$ T cells ($p<0.5$, Fig. 32 A and B).

Surprisingly, autologous and allogeneic co-cultures between mDC LPS and $CD4^+$ T cells displayed a comparable percentage of cell conjugates (Fig. 31 C and D), indicating that sustained MHC-TcR recognition at the contact zone did not affect the number of conjugates detected. In addition, the frequency of mDC–$CD4^+$ T-cell conjugates increased over time in both autologous and allogeneic co-cultures (Fig. 33 A and B), regardless of the presence or absence of HIV-1. These findings indicated that, contrary to the virological synapse, the presence of HIV-1 did not modulate the formation of conjugates comprising mDC LPS harboring HIV-1 and uninfected primary $CD4^+$ T cells.

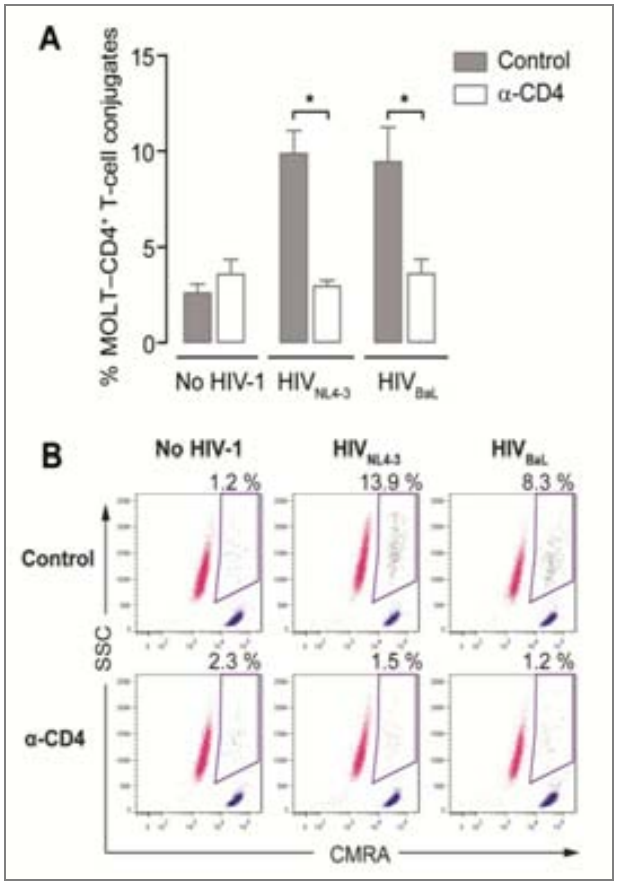


**Figure 32. Conjugate formation comprising productively infected cells and uninfected primary CD4$^+$ T cells is dependent on the interaction between the viral Env on the surface of the HIV-1–infected cell and its primary receptor, CD4, on the target cell. (A)** Comparative quantification of cellular conjugates between the MOLT cell lines chronically infected with X4- ($MOLT_{NL4-3}$) or R5-tropic ($MOLT_{BaL}$) HIV-1 and non-activated primary CD4$^+$ T cells after 2 hours of co-culture. Conjugate formation between HIV-1–infected and uninfected T cells was abrogated by blocking gp120 binding to CD4. **(B)** Representative experiment of quantification of cellular conjugates between HIV-1–infected MOLT cells and non-activated CMRA-labeled CD4$^+$ T cells by flow cytometry; cellular conjugates are shown in black, MOLT cells in red, and CMRA-labeled CD4$^+$ T cells in blue. Numbers represent the percentage of cellular conjugates in the gate. Asterisks indicate significant differences compared with co-cultures in the absence of HIV-1 ($p<0.05$, Wilcoxon matched-pairs test). Data are expressed as mean and SEM from three independent experiments including cells from six different donors.

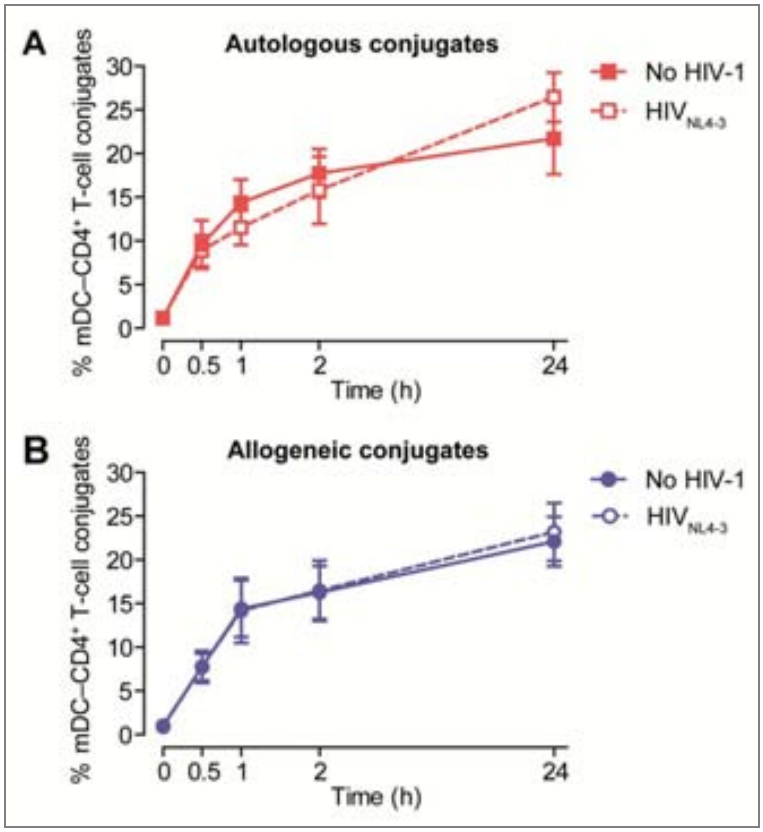


**Figure 33. Sustained MHC-TcR recognition at the contact zone between mDC LPS and CD4$^+$ T cells does not affect the number of conjugates.** Kinetics of conjugate formation in autologous **(A)** or allogeneic **(B)** mDC–CD4$^+$ T-cell co-cultures in the presence or absence of HIV-1. The percentage of cellular conjugates increased over time independently of the presence of HIV-1 in both autologous and allogeneic co-cultures. No statistically significant differences were observed between autologous co-cultures **(A)** and allogeneic **(B)** co-cultures. Data are expressed as mean and SEM from three independent experiments including cells from six different donors.

## 2. Blocking of ICAM-1 and/or LFA-1 impairs the formation of mDC–CD4$^+$ T cell conjugates

Since we observed that HIV-1 and antigen recognition appeared to exercise albeit minimal control over the interaction between mDC LPS and CD4$^+$ T cells, we analyzed the role of adhesion molecules during formation of mDC–CD4$^+$ T-cell conjugates (Fig.22 A and Fig. 34 A). Engagement of ICAM-1 and LFA-1 is considered essential for the formation of DC–T cell immunological synapses [265] and the infiltration of lymphocytes into sites of inflammation [266]. Therefore, we specifically evaluated the role of these adhesion molecules in the formation of mDC–CD4$^+$ T-cell conjugates by using blocking mAb against these adhesion molecules. We observed

that treatment of both mDC LPS and $CD4^+$ T cells with α-LFA-1 (mAb 68.5A5) reduced the frequency of cellular conjugates by 45% ($p<0.05$, Fig. 34 B), whereas blocking of ICAM-1 with mAb RM3A5 inhibited the formation of autologous and allogeneic conjugates by 60% ($p<0.05$, Fig. 34 B). However, we did not observe a synergistic effect when ICAM-1 on mDC and LFA-1 on $CD4^+$ T cells were blocked, and vice versa ($p<0.05$, Fig. 34 B). Moreover, the addition of blocking α-ICAM-3 mAb to both mDC LPS and $CD4^+$ T cells failed to inhibit the formation of cellular conjugates (Fig. 34 B). Despite the relevance of the CD4 receptor in the formation of the virological synapse (Fig. 32) [176, 191, 192], the blockade of the CD4 molecule with mAb Leu3a did not have any significant impact on the formation of mDC–$CD4^+$ T-cell conjugates (Fig. 34 B).

Engagement of CD4 by Env at the virological synapse between infected and uninfected $CD4^+$ T cells triggers actin-dependent recruitment of HIV-1 receptors and adhesion molecules to the contact interface, thus stabilizing the adhesive interactions and enabling the final transfer of HIV-1 to the target cell [176, 191, 192]. Consequently, we analyzed whether the cytoskeleton was necessary for the establishment of the mDC–T-cell interaction. Addition of cytochalasin D effectively blocked the formation of mDC–$CD4^+$ T-cell conjugates ($p<0.05$) (Fig. 34 B), indicating that this process requires an active actin cytoskeleton to rearrange receptors towards the interface of the mDC–T-cell contact. Conversely, the presence of bacterial superantigen (SEA) in the co-culture did not increase the percentage of cellular conjugates (Fig. 34 B), probably because the SEA induced long-lasting interactions between mDC and T cells, thus enabling the formation of more stable conjugates and the subsequent functional maturation of the immunological synapse [267]. As shown in Fig. 31 and Fig. 33, both autologous and allogeneic co-cultures yielded similar percentages of cellular conjugates and were equally susceptible to the blocking reagents used in these experiments (Fig. 34 B), thus confirming that neither antigen recognition nor sustained MHC-TcR interaction alone enhanced conjugate formation. Taken together, these data suggest that the adhesion molecules ICAM-1 and LFA-1 are the main driving force in modulating the formation of mDC–$CD4^+$ T-cell conjugates and could play a key role in transmission of HIV-1 across the infectious synapse.

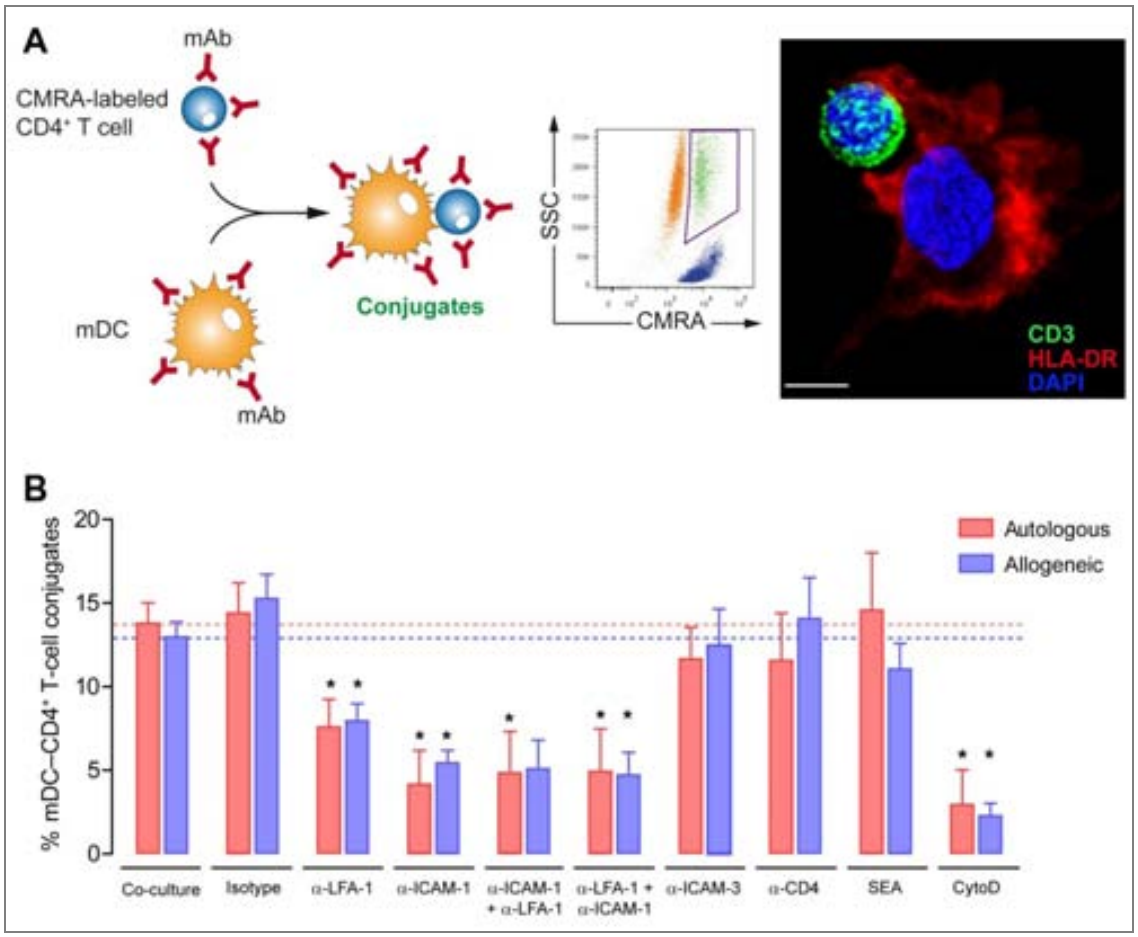


**Figure 34. Blocking of ICAM-1 and LFA-1 impairs conjugate formation between mDC LPS and primary CD4$^+$ T cells. (A)** ***Left.*** Experimental procedure for quantification of cell conjugates between mDC LPS and CMRA-labeled CD4$^+$ T cells in the presence or absence of several reagents. Cell conjugates were analyzed by flow cytometry as in Fig. 31 B. ***Right.*** Phenotype of mDC–CD4$^+$ T cell contact by confocal microscopy. HLA-DR and CD3 molecules were stained to unequivocally identify the mDC LPS and the CD4$^+$ T cell, respectively. Confocal Z-stacks were acquired every 0.25 µm steps and processed with Volocity 6.1 software (Improvision, PerkinElmer) using the maximum fluorescent intensity projection for HLA-DR and CD3 stainings and the isosurface modeling for DAPI-stained nucleus (scale bar: 5 µm). **(B)** Comparative quantification of cellular conjugates after 2 hours of autologous co-cultures (red bars) or allogeneic co-cultures (blue bars) between mDC LPS and non-activated CD4$^+$ T cells. Blocking of LFA-1 and ICAM-1 significantly inhibited the formation of autologous and allogeneic conjugates. Both autologous and allogeneic conjugates were equally affected by the blocking reagents. No differences were detected between autologous and allogeneic co-cultures. The α-ICAM-1 + α-LFA-1 condition represents separate pre-incubation of mDC LPS with α-ICAM-1 mAb and CD4$^+$ T cells with α-LFA-1 mAb, before launching co-culture, while the α-LFA-1 + α-ICAM-1 condition designates separate pre-incubation of mDC LPS with α-LFA-1 mAb and CD4$^+$ T cells with α-ICAM-1 mAb. Asterisks indicate significant differences compared with negative controls ($p<0.05$, Wilcoxon matched-pairs test); mAb conditions were compared with an isotype control, whereas SEA and cytochalasin D were compared with the medium. Data are expressed as mean and SEM from at least three independent experiments including cells from at least six different donors.

### 3. mDC-mediated HIV-1 *trans*-infection of primary CD4$^+$ T cells is dependent on the interaction between ICAM-1 and LFA-1 and is enhanced by antigen recognition

Once we confirmed that ICAM-1 and LFA-1 and not HIV-1 or antigen recognition modulate the formation of mDC–CD4$^+$ T-cell conjugates, we investigated the role of these factors in mDC-mediated HIV-1 *trans*-infection to primary CD4$^+$ T cells. We performed both autologous and allogeneic co-cultures of mDC LPS pulsed with the reporter virus $HIV_{NL4\text{-}3Ren}$ and non-activated primary CD4$^+$ T cells in the presence of several blocking reagents (Fig. 19). In this set of experiments, a co-culture performed with the protease inhibitor SQV allowed us to confirm net *trans*-infection, thus avoiding re-infection events between T cells (Fig. 35), while a control co-culture with AZT, which completely blocked luciferase activity, confirmed productive HIV-1 replication in co-cultures.

Allogeneic conjugates between HIV-1–pulsed mDC LPS and non-activated primary CD4$^+$ T cells led to a three-fold increase in viral replication than autologous co-cultures ($p<0.05$, Fig. 35 A and B). This observation suggests a role for antigen recognition in HIV-1 replication in CD4$^+$ T lymphocytes, probably because of an increase in cell activation mediated by contact with mDC LPS. Consistent with this hypothesis, we observed a dramatic increase in HIV-1 replication as a result of mDC-mediated *trans*-infection when MHC-TcR interactions were stabilized with SEA ($p<0.05$, Fig. 35 A and B), which corresponds to one cycle of infection (Fig. 36 A). Interestingly, direct infection of non-activated primary CD4$^+$ T cells by free HIV-1 was almost null, even under α-CD3 (mAb OKT3) or SEA activation conditions (Fig. 36 B). This could be explained because SEA needs to simultaneously bind to MHC class II molecule on the APC and to the TcR on the CD4$^+$ T cell to lead a non-specific stimulation of T lymphocytes [268, 269]. Meanwhile, activation of T cells via CD3 needs costimulatory signaling provided by APC. Therefore, the activation and subsequent HIV-1 infection of CD4$^+$ T cell cultured with SEA and α-CD3 depends on the presence of an APC, in this case mDC LPS, which provides MHC or costimulatory signaling. Consequently, the increase in the mDC-mediated *trans*-infection, either in autologous or allogeneic co-cultures, in α-CD3 and SEA conditions is dependent of the presence of mDC LPS.

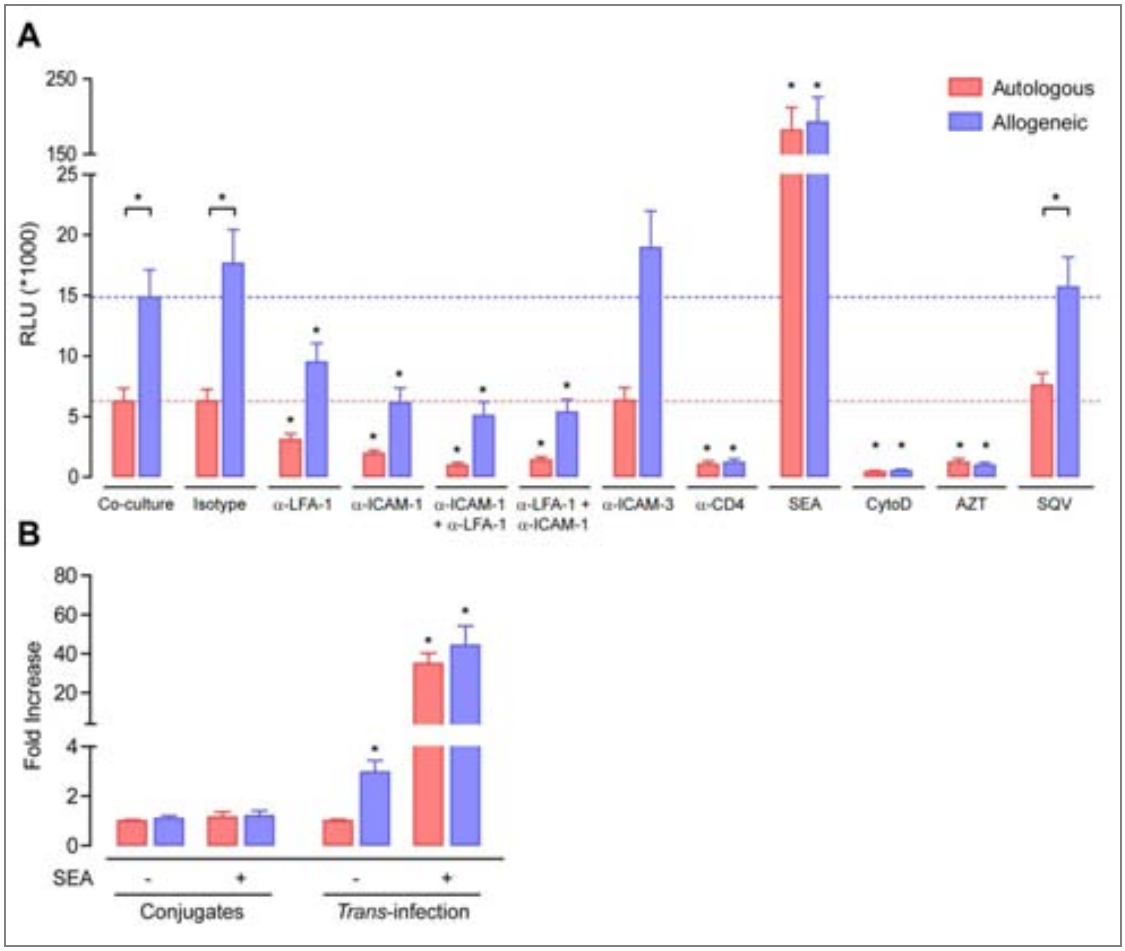


**Figure 35. mDC-mediated HIV-1 *trans*-infection to primary CD4$^+$ T cells is dependent on the interaction between ICAM-1-LFA-1 and is enhanced by antigen recognition. (A)** Comparative HIV-1 mDC-mediated *trans*-infection to non-activated CD4$^+$ T cells in autologous or allogeneic co-cultures in the presence of several reagents. Allogeneic co-cultures (blue bars) led to a three-fold increase in productive HIV-1 replication compared with autologous co-cultures (red bars) ($p<0.05$, Wilcoxon matched-pairs test). Blocking ICAM-1 and LFA-1 with mAb significantly inhibited the mDC-mediated HIV-1 *trans*-infection. The α-ICAM-1 + α-LFA-1 condition represents separate pre-incubation of mDC LPS with α-ICAM-1 mAb and CD4$^+$ T cells with α-LFA-1 mAb, before launching co-culture, whereas the α-LFA-1 + α-ICAM-1 condition designates separate pre-incubation of mDC LPS with α-LFA-1 mAb and CD4$^+$ T cells with α-ICAM-1 mAb. Asterisks indicate significant differences compared with negative controls ($p<0.05$, Wilcoxon matched-pairs test); mAb conditions were compared with the isotype control, whereas AZT, SQV, SEA and cytochalasin D were compared with the medium. Data are expressed as mean and SEM from three independent experiments including cells from at least six different donors. RLU, relative light units. **(B)** Fold increase of conjugate formation and mDC-mediated HIV-1 *trans*-infection in autologous and allogeneic mDC–CD4$^+$ T cell co-cultures in the presence or absence of SEA. All conditions were compared to autologous co-cultures without SEA, shown as unity in the plot.

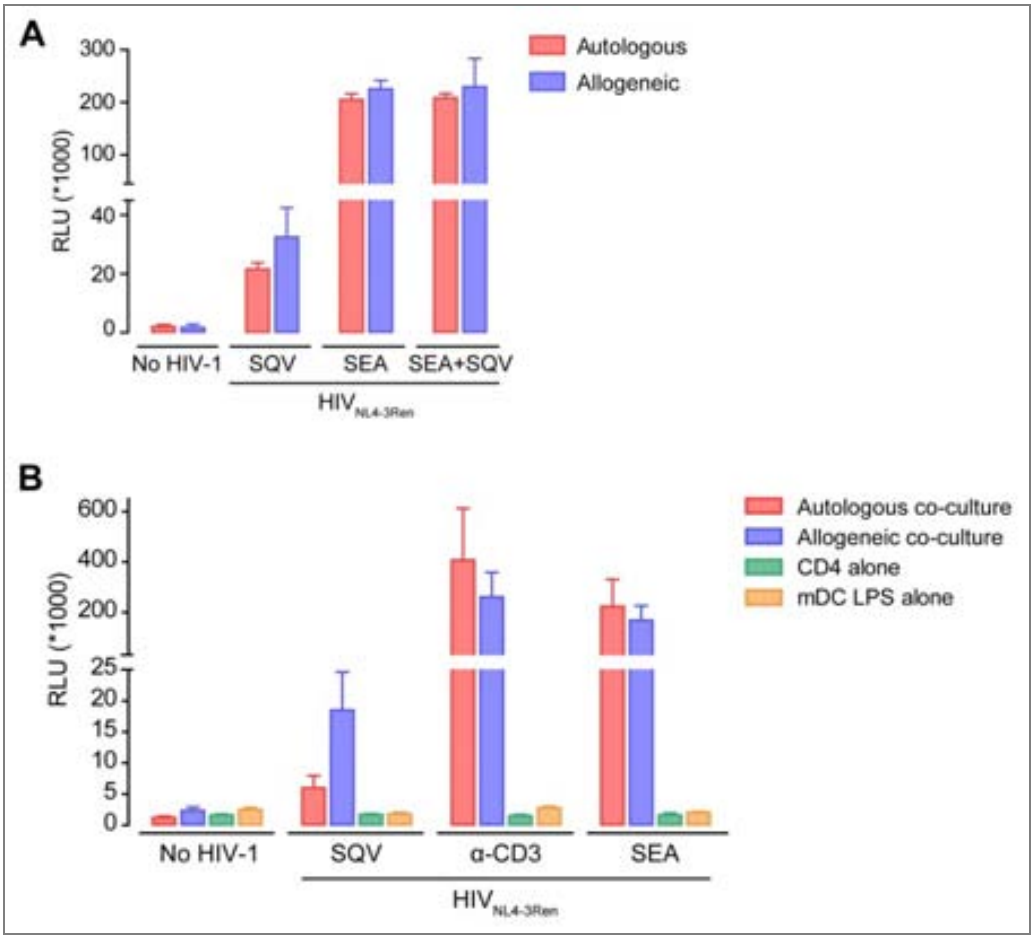


**Figure 36. The productive infection of primary CD4$^+$ T cells is enhanced through MHC and costimulatory signaling mediated by mDC LPS. (A)** mDC-mediated HIV-1 *trans*-infection in autologous and allogeneic mDC–CD4$^+$ T cell co-cultures with SEA, also in the presence or absence of SQV. Data are expressed as mean and SEM from one experiments including cells from at least two different donors. **(B)** Evaluation of direct infection by free virus of non-activated primary CD4$^+$ T cells and mDC cultured alone compared with HIV-1 mDC-mediated *trans*-infection to CD4$^+$ T cells (autologous and allogeneic co-cultures). Data are expressed as mean and SEM from one experiments including cells from at least two different donors. RLU, relative light units.

Some reports have already illustrated the importance of the interaction between ICAM-1 and LFA-1 in facilitating transmission of HIV-1 between DC and target cells [186, 200, 201]. Furthermore, ICAM-1 is the main force driving formation of mDC–CD4$^+$ T-cell conjugates (Fig. 34). As expected, blockade of ICAM-1 with the mAb RM3A5 not only inhibited the formation of mDC–CD4$^+$ T-cell conjugates (Fig. 34 B), but also impaired the mDC-mediated *trans*-infection of HIV-1 to CD4$^+$ T lymphocytes by 60% to 70% ($p<0.05$, Fig. 35 A). Productive infection of CD4$^+$ T cells was also significantly abrogated by 45 to 50% when cells were treated with the mAb α-LFA-1 68.5A5 ($p<0.05$, Fig. 35 A). In addition, when both ICAM-1 and LFA-1 were simultaneously blocked, inhibition of mDC-mediated *trans*-infection of HIV-1 to primary CD4$^+$ T cells

reached 75% to 90% in both autologous and allogeneic co-cultures ($p<0.05$, Fig. 35 A). Consequently, blocking of ICAM-1–LFA-1 interactions between mDC LPS and non-activated primary $CD4^+$ T cells substantially reduced the productive mDC-mediated HIV-1 *trans*-infection of $CD4^+$ T cells. In addition, treatment with α-ICAM-3 did not reduce viral replication in target cells (Fig. 35 A), thus confirming ICAM-3–independent mDC-mediated transmission of HIV-1 to primary $CD4^+$ T cells [201]. On the other hand, although blocking the CD4 receptor did not affect the formation of cellular conjugates (Fig. 35 B), the addition of α-CD4 mAb Leu3a successfully inhibited productive infection of $CD4^+$ T cells through mDC-mediated *trans*-infection of HIV-1 by hampering binding of HIV-1 Env to the CD4 molecule in target cells ($p<0.05$, Fig. 35 A). In addition, we confirmed that DC-mediated transmission of HIV-1 to primary $CD4^+$ T cells was also dependent on the integrity of the actin cytoskeleton, since cytochalasin D impaired recruitment of receptors to the infectious synapse ($p<0.05$, Fig. 35 A). Taken together, these findings indicate that the interactions between ICAM-1 and LFA-1 in mDC LPS and primary $CD4^+$ T cells, as well as a functional actin cytoskeleton, were necessary for efficient mDC-mediated HIV-1 *trans*-infection in an antigen-dependent and antigen-independent manner across the infectious synapse. Importantly, our data suggest that the role of antigen recognition in *trans*-infection of HIV-1 is restricted to late synaptic events, since cellular conjugation is unaffected by allogeneic or SEA stimulation, whereas productive infection of T cells is greatly enhanced (Fig. 35 B).



## 4. Antigen-recognition, but not HIV-1, enhances $CD4^+$ T-cell activation and proliferation

HIV-1 replication is highly dependent on the activation status of target cells, since highly activated T lymphocytes are the main target for viral spread and consequent cell destruction [270, 271]. To elucidate the level of activation in primary $CD4^+$ T cells after antigen-dependent and antigen-independent contacts with mDC LPS, we evaluated surface expression of the activation markers CD69 and CD25 after 16 hours and 5 days of autologous and allogeneic co-cultures. We also monitored the T-cell proliferation induced after 5 days of co-culture using a flow cytometry assay based on carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). Simultaneously, we assessed the effect on these parameters induced by the presence of HIV-1 in the co-culture. $CD4^+$ T cells were distinguished from mDC LPS in co-cultures by gating

cellular singlets and selecting the CD2-positive, CD11c-negative population; surface activation markers and proliferation were then analyzed within lymphocytes (Fig. 23). As positive controls, SEA or mAb α-CD3 OKT3 were added to mDC–$CD4^+$ T-cell co-cultures.

Expression levels of CD69 and CD25 increased over time in $CD4^+$ T cells cultured with allogeneic mDC, as compared with autologous co-cultures ($p<0.05$, Fig. 37 A and B). Consequently, the antigen-dependent HIV-1 infection and virus replication we observed in Fig. 35 could be associated with the higher immune activation of $CD4^+$ T cells mediated by allogeneic mDC LPS. Interestingly, similar levels of CD69 and CD25 expression were detected in $CD4^+$ T cells cultured with HIV-1–pulsed or –unpulsed mDC, both in autologous and in allogeneic co-cultures (Fig. 37 A and B). This finding contrasts with the results of other studies that reveal a substantial Nef-dependent increase in CD69 expression in T cells co-cultured with HIV-1–infected immature DC [272]. Since the $HIV_{NL4\text{-}3Ren}$ used in our experiments lacks the gene *nef*, the $CD4^+$ T-cell activation in our experimental setting should be driven by the contacts with mDC LPS. Furthermore, in our experiments we used DC LPS expressing high levels of MHC and co-stimulatory molecules at the cell membrane [as observed in Fig. 25 and [273]], with competence to stimulate T cells and poor ability to support viral replication [144].

After 5 days of co-culture, we examined the T-cell proliferation induced by the autologous and allogeneic contacts with mDC LPS in the presence or absence of HIV-1 by analyzing the percentage of the $CD4^+$ T cells divided (Fig. 38 A) and the average number of divisions in the responding cells (proliferation index, Fig.38 B). More $CD4^+$ T cells from allogeneic co-cultures than from autologous co-cultures divided at least once in the presence of HIV-1 ($p<0.05$, Fig. 38 A), although this trend was also observed in the absence of HIV-1. However, the responding T lymphocytes from autologous and allogeneic co-cultures underwent the same number of proliferation cycles, independently of HIV-1 (Fig. 38 B). Consequently, the mixed lymphocyte reaction (MLR) between allogeneic mDC LPS and $CD4^+$ T cells activated more lymphocytes to proliferate, but responding cells supported the same number of cell divisions. These data strongly suggest that immune activation of T cells mediated by mDC can provide an environment to facilitate HIV-1 transmission and replication. A more sustained MHC-TcR interaction due to antigen-dependent contacts between APC and T cells allows higher lymphocyte activation and, consequently, increased susceptibility to infection of target cells. Therefore, this observation is consistent with the findings that

antigen-specific $CD4^+$ T cells are preferentially infected by HIV-1 *in vivo*, resulting in depletion of responding $CD4^+$ T lymphocytes and loss of immunological control of HIV-1 replication [274-276].

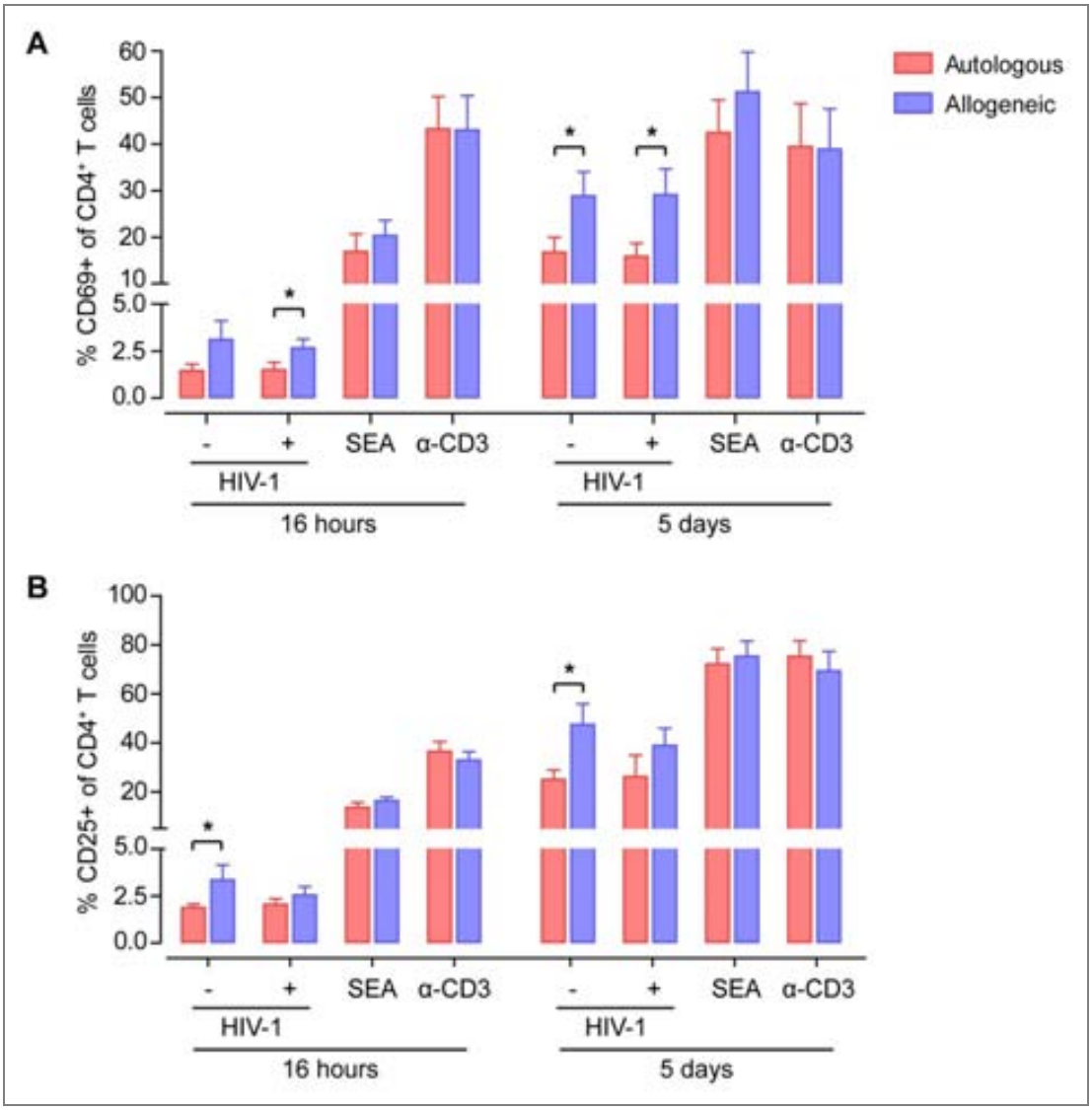


**Figure 37. $CD4^+$ T-cell activation is enhanced by antigen recognition but not by the presence of HIV-1. (A)** CD69 expression in $CD4^+$ T cells after co-culture for 16 hours or 5 days with autologous mDC LPS (red bars) or allogeneic mDC LPS (blue bars) in the presence or absence of HIV-1. The expression of CD69 surface marker was higher in $CD4^+$ T cells co-cultured with allogeneic mDC LPS. No differences were observed in the presence of HIV-1 compared with control co-cultures without virus. **(B)** CD25 expression in $CD4^+$ T cells after co-culture for 16 hours or 5 days with autologous mDC LPS (red bars) or allogeneic mDC LPS (blue bars) in the presence or absence of HIV-1. Stimulation with allogeneic mDC LPS induced higher levels of CD25 expression in $CD4^+$ T lymphocytes ($p<0.05$). The presence of HIV-1 did not exert a detectable effect on CD25 expression. Positive controls consisting of co-cultures with SEA or α-CD3 are shown. Data are expressed as mean and SEM from three independent experiments including cells from at least six different donors. Asterisks denote significant differences ($p<0.05$, Wilcoxon matched-pairs test).

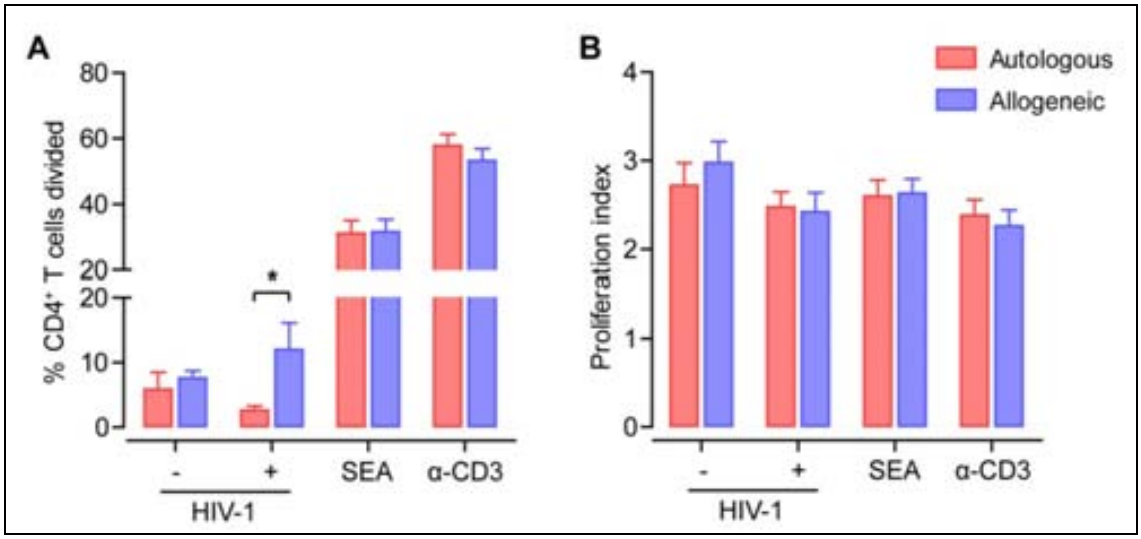


**Figure 38. CD4$^+$ T-cell proliferation is enhanced by antigen recognition but not by the presence of HIV-1.** Percentage of CD4$^+$ T cells divided **(A)** and proliferation index of CD4$^+$ T cells (number of cell divisions/number of divided cells) **(B)** after co-culture for 5 days with autologous mDC LPS (red bars) or allogeneic mDC LPS (blue bars) in the presence or absence of HIV-1. Allogeneic co-cultures exhibited more CD4$^+$ T-cell proliferation than autologous co-cultures, although no significant differences were observed with regard to the presence or absence of HIV-1. Positive controls consisting of co-cultures with SEA or α-CD3 are shown. Data are expressed as mean and SEM from three independent experiments including cells from at least six different donors. Asterisks denote significant differences ($p<0.05$, Wilcoxon matched-pairs test).

# Chapter 6

## RESULTS III

*Sialyllactose in viral membrane gangliosides is a novel molecular recognition pattern for mDC LPS capture of HIV-1*

*This chapter is part of the manuscript:*

Izquierdo-Useros N., Lorizate M., Contreras F-X., **Rodriguez-Plata M.T.**, Glass B., Erkizia I., Prado J.G., Casas J., Fabriás G., Kräusslich H-G., Martinez-Picado J. **Sialyllactose in viral membrane gangliosides is a novel recognition molecular pattern for mature dendritic dell capture of HIV-1.** *PLoS Biology*. 2012;10(4):e1001315. *Second authorship*.

HIV-1 Env provides the primary mechanism for HIV-1 fusion and entry by binding with CD4 and coreceptor of host cell. In addition, HIV-1 Env can attach to other factors expressed by DC, such as DC-SIGN [76], syndecan-3 [167], DCIR [157] and GalCer [168]. However, it has been described an HIV-1 gp120-independent mechanism of viral binding and uptake that is upregulated upon DC maturation [169, 170], although the molecular mechanism underlying this Env-independent HIV-1 uptake by mDC remains largely uncharacterized. HIV-1 is known to bud from cell membrane microdomains, named lipid rafts [277], which are enriched in cholesterol, sphingomyelin and GSL [278-281]. Interestingly, previous works have reported that treatment of producer cells of HIV-1 or HIV-1 Gag enhanced green fluorescent protein (eGFP)–expressing virus-like particles ($VLP_{HIV\text{-}Gag\text{-}eGFP}$) with inhibitors of GSL biosynthesis yielded particles with less GSL, which exhibited reduced entry into mDC LPS [171, 172], without affecting net release from producer cells [282]. Consequently, as GSL are a major component of lipid rafts, it suggests that the HIV-1 gp120-independent mechanism for viral capture by DC could be mediated by a host-derived GSL that is incorporated into the virion as it buds from producer cell.



In this study, we sought to investigate the molecular determinants involved in HIV-1 Env-independent binding and internalization mediated by mDC LPS. Using liposomes to mimic the lipid composition and size of HIV-1, we demonstrated that gangliosides are the key molecules that mediate liposome uptake. We extended these observations to virus-like particles (VLP) and HIV-1, characterizing a new role for these GSL as viral attachment factors. Furthermore, we identified sialyllactose on HIV-1 membrane gangliosides as a novel molecular recognition pattern that mediates virus uptake into mDC LPS.

## 1. Gangliosides are required for viral capture mediated by mDC LPS

Plasma membrane GSL on the target cell have been proposed to significantly contribute to HIV-1 pathogenesis in multiple ways: as an alternate receptor for CD4-independent entry in several cell types; by allowing HIV-1 transcytosis; or by stabilizing gp120-gp41-mediated membrane fusion [283]. GSL are members of the family of membrane-associated ceramide (Cer) sphingolipids, which are built on a Cer lipid moiety that consists of a sphingosine coupled with a fatty acid. The Cer backbone is then modified to become sphingomyelin, sulfatides, or GalCer [283, 284]. Although they only count for the 5% of the overall membrane lipid composition, GSL are highly enriched in lipid raft plasma membrane domains [278, 280, 281] from where HIV-1 is thought to bud [277, 279]. Considering this, we investigated the potential role of GSL in the membrane of HIV-1 and its impact on the viral capture by mDC LPS. The major GSL components in human $CD4^+$ T cells are GM1 and GM3 gangliosides [285], which are characterized by one sialic acid linked on the sugar chain of the GSL. Interestingly, the ganglioside GM3 was previously identified in the membrane of different retroviruses including HIV-1 [286, 287]. We were able to confirm the presence of GM3 in $HIV_{NL4-3}$ derived from the T-cell line MT-4 by mass spectrometry (Fig. 39 A). In addition, we detected several other gangliosides including GM1, GM2, and GD1 in the HIV-1 membrane (Fig. 39 A).

To test whether gangliosides in the outer leaflet of HIV-1 or vesicular membranes can act as attachment factors yielding mDC LPS uptake, Texas Red (tRed)-labeled large unilamellar vesicles (LUV) mimicking the size and lipid composition of HIV-1 were prepared (referred to as $LUV_{HIV\text{-}tRed}$ and prepared as in [288]). $LUV_{HIV\text{-}tRed}$ contained different gangliosides (Fig. 39 B), with equal fluorescence intensities. mDC LPS were pulsed with either $LUV_{HIV\text{-}tRed}$ or VLP for 4 hours at 37ºC in 5% $CO_2$ and the percentage of fluorescent cells was determined by FACS. Similar to our previous results with infectious HIV-1 [169], a high percentage of mDC LPS captured the fluorescent $VLP_{HIV\text{-}Gag\text{-}eGFP}$ (Fig. 39 C). On the other hand, no significant uptake into mDC LPS was observed for $LUV_{HIV\text{-}tRed}$, which contained the main lipid constituents of HIV-1, but were devoid of gangliosides ($p$<0.0001, paired t test) (Fig. 39 C). Uptake into mDC LPS remained negative for $LUV_{HIV\text{-}tRed}$ containing Cer ($p$<0.0001, paired t test) (Fig. 39 C). This was completely different when monosialogangliosides such as GM3, GM2, or GM1a were incorporated into the LUV; mDC LPS were able to capture these

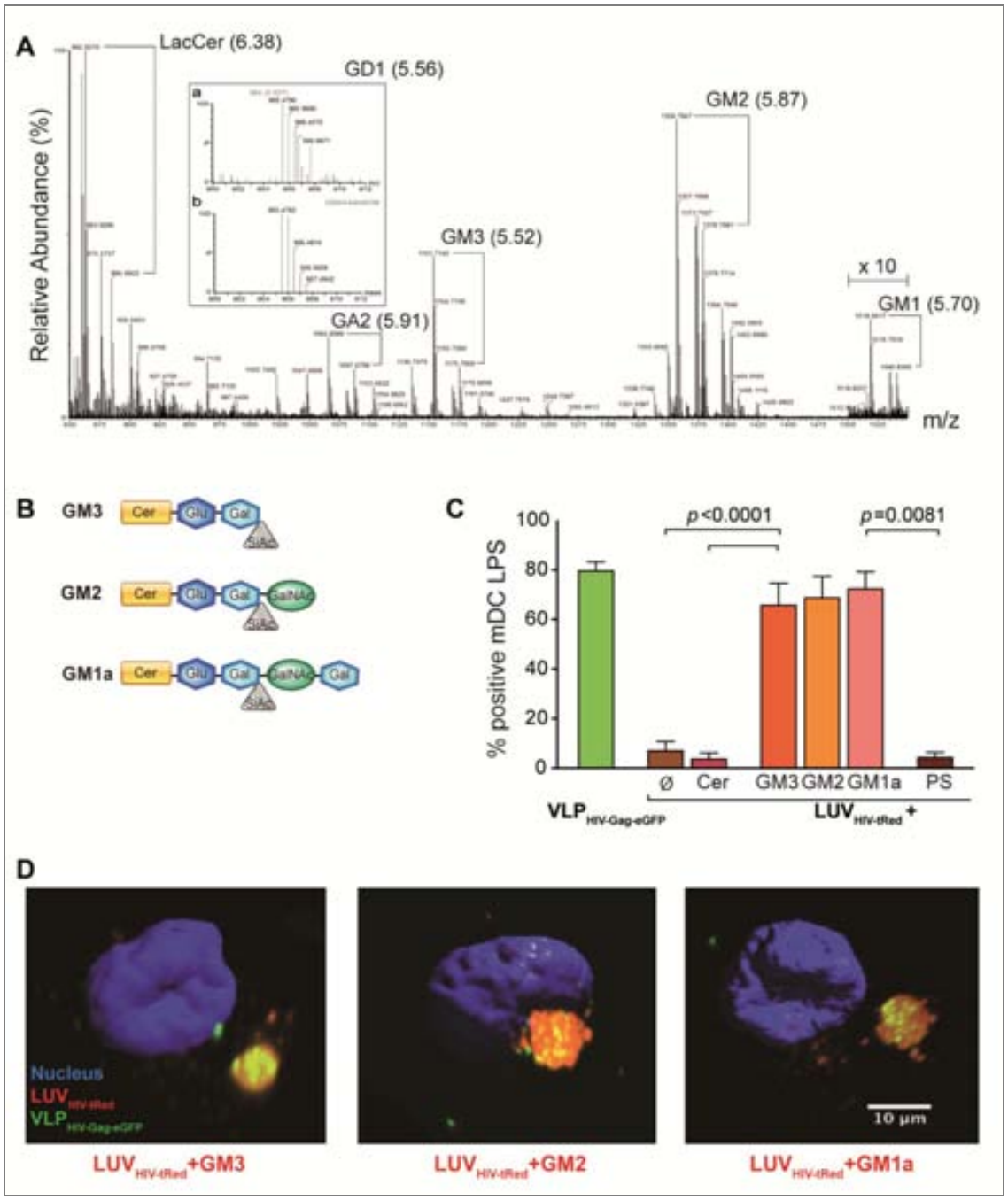


**Figure 39. Gangliosides are required for viral capture mediated my mDC LPS. (A)** Ganglioside detection in lipid extracts from MT4 derived $HIV_{NL4-3}$. Partial mass spectrum (from 850 to 1550 amu) corresponding to the 5.3- and 6.5-min range of a UPLC/TOF ESI (+) chromatogram representative of three different viral isolations. The selected time range corresponds to the N-hexadecanoyl (N-C16) species. The N-C22, N-C24, and N-C24:1 species were also observed. For each compound of interest identified, the exact mass of its [M+H]+ and [M+Na]+ ions are indicated. The retention time of each compound is given within parenthesis next to its abbreviation. Inset: exact mass ion cluster obtained at 5.56 min for GD1 (a) and exact mass ion cluster corresponding to the formula C82H144N4O39 with a charge state of 2 (b). X 10 indicates a ten-fold magnification between 1,500 and 1,550 amu. GA2, Nacetyl-D-galactosaminyl-D-galactosyl-D-glucosylceramide; LacCer, D-galactosyl-D-glucosylceramide (lactosylceramide). **(B)** Schematic view of the gangliosides present in the LUV employed for the experiment C. **(C)** Comparative mDC LPS capture of $VLP_{HIV-Gag-eGFP}$ produced in HEK-293T and

distinct fluorescent $LUV_{HIV\text{-}tRed}$ containing Cer, GM3, GM2, GM1a, or phosphatidylserine (PS). A total of 2 x $10^5$ mDC LPS were pulsed for 4 hours at 37ºC in $CO_2$ with 100 µM of LUV or 75 ng of $VLP_{HIV\text{-}Gag\text{-}eGFP}$ Gag in 0.2 ml, washed with PBS, and assessed by FACS to obtain the percentage of tRed or eGFP-positive cells. Data show mean values and standard error of the mean (SEM) from five independent experiments including cells from at least six donors. mDC LPS capture significantly higher amounts of GM3-containing $LUV_{HIV\text{-}tRed}$ than Cer or Ø $LUV_{HIV\text{-}tRed}$ ($p$<0.0001, paired t test). mDC LPS capture significantly higher amounts of GM1a-containing $LUV_{HIV\text{-}tRed}$ than negatively charged PS-$LUV_{HIV\text{-}tRed}$ ($p$=0.0081, paired t test). **(D)** Confocal microscopy analysis of mDC LPS previously pulsed with 100 µM of GM1a, GM2, and GM3 containing $LUV_{HIV\text{-}tRed}$ and then exposed to 75 ng of $VLP_{HIV\text{-}Gag\text{-}eGFP}$ Gag. 3-D reconstructions of the *x-y* sections collected throughout the whole mDC LPS *z* volume every 0.1 µm. Isosurface representation of DAPI stained nucleus is shown, computing the maximum intensity fluorescence of the sac-like compartment surface within a 3-D volumetric *x-y-z* data field, where $VLP_{HIV\text{-}Gag\text{-}eGFP}$ and ganglioside-containing $LUV_{HIV\text{-}tRed}$ are accumulated within the same compartment.



liposomes with equal efficiency as $VLP_{HIV\text{-}Gag\text{-}eGFP}$ (Fig. 39 C). To ensure that this capture was not merely due to electrostatic interactions between negatively charged gangliosides and surface charges on mDC LPS, $LUV_{HIV\text{-}tRed}$ containing negatively charged phosphatidylserine (PS) were analyzed in parallel and were found to be negative for mDC LPS capture ($p$=0.0081, paired t test) (Fig. 39 C). These results reveal that monosialogangliosides mediate LUV capture by mDC LPS, and that the carbohydrate head group is essential for this process.

To further address whether the ganglioside-mediated LUV capture reach the same compartment that $VLP_{HIV\text{-}Gag\text{-}eGFP}$ in mDC LPS, uptake of GM-containing $LUV_{HIV\text{-}tRed}$ and $VLP_{HIV\text{-}Gag\text{-}eGFP}$ by mDC LPS was monitored by confocal microscopy. mDC LPS were pre-incubated 3 hours at 37ºC in $CO_2$ with GM-containing $LUV_{HIV\text{-}tRed}$ and subsequently incubated with $VLP_{HIV\text{-}Gag\text{-}eGFP}$ for three additional hours. Confocal microscopy of fixed cells revealed that GM-containing $LUV_{HIV\text{-}tRed}$ and VLP polarized towards the same cell area in mDC LPS (Fig. 39 D), concentrating them in a large sac-like compartment, as previously described for HIV-1 [197]. Furthermore, VLP extensively co-localized with GM-containing $LUV_{HIV\text{-}tRed}$ (including either GM1a, GM2, or GM3) in the same intracellular compartment (Fig. 39 D).

## 2. Ganglioside complexity determines mDC LPS capture

To gain further insight into the molecular determinant structure required for efficient recognition by mDC LPS, $LUV_{HIV\text{-}tRed}$ carrying more complex gangliosides were produced, including two, three, and four sialic acid groups at diverse positions in the carbohydrate polar head group (di-, tri-, and tetra-sialogangliosides) (Fig. 40). mDC LPS pulsed with an equal amount of $LUV_{HIV\text{-}tRed}$-containing gangliosides with two or three sialic acids (GD1b and GT1b, respectively) captured these particles with the same efficiency as GM1a-$LUV_{HIV\text{-}tRed}$ (Fig. 40). However, capture was almost completely lost for $LUV_{HIV\text{-}tRed}$ containing a ganglioside with four sialic acids (GQ1b) (Fig. 40). These results indicate that complex gangliosides with up to three sialic acids located in distinct positions of the carbohydrate head group share a common structure determinant for mDC LPS uptake, which is lost in GQ1b.

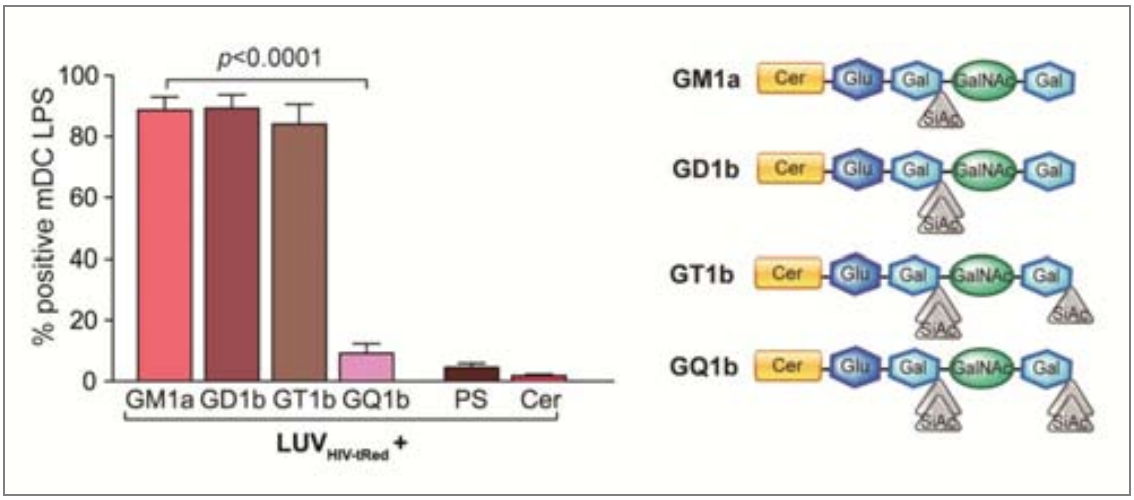


**Figure 40. Ganglioside complexity determines mDC LPS capture.** Comparative mDC LPS capture of distinct $LUV_{HIV\text{-}tRed}$ containing GM1a, polysialogangliosides such as GD1b, GT1b, and GQ1b; phosphatidylserine (PS) and Cer. A total of 2 x $10^5$ mDC LPS were pulsed for 4 hours at 37ºC with 100 µM of LUV, washed with PBS, and assessed by FACS to obtain the percentage of tRed-positive cells. Data show mean values and SEM from two independent experiments including cells from six donors. mDC LPS capture significantly higher amounts of GM1a-containing $LUV_{HIV\text{-}tRed}$ than GQ1b-containing $LUV_{HIV\text{-}tRed}$ ($p$<0.0001, paired t test). Schematic view of the gangliosides present in the LUV employed for these experiments is shown on the right.

### 3. Identification of the molecular recognition domain present in gangliosides that is essential for mDC LPS capture

The lack of internalization of Cer-containing LUV indicated that the carbohydrate head group is specifically required for mDC LPS capture. Sialic acid has previously been identified as a cellular receptor for certain viruses [289]. We therefore tested its importance for mDC LPS capture. Incubation of mDC LPS with equal concentrations of $LUV_{HIV\text{-}tRed}$ containing Cer, GM1a, or GM1 without the sialic acid group (Asialo GM1) revealed sialic acid-dependent capture (Fig. 41 A). Thus, the sialic acid moiety in gangliosides is necessary for specific recognition by mDC LPS. To assess the contribution of other components of the carbohydrate head group, we prepared $LUV_{HIV\text{-}tRed}$ containing either GM4 (lacking the glucose moiety of GM3) (Fig. 41 B) or GalCer (lacking both the glucose and sialic acid moieties of GM3) (Fig. 41 B). mDC LPS incubated with GM4- or GalCer-containing $LUV_{HIV\text{-}tRed}$ showed only background levels of liposome capture (Fig. 41 C), indicating that the glucose moiety of sphingolipids is also necessary for mDC LPS capture. Given that the carbohydrate moiety within gangliosides constitutes the molecular recognition determinant for mDC LPS capture, these head groups should compete for VLP and LUV uptake. Capture of GM3-containing $LUV_{HIV\text{-}tRed}$ or $VLP_{HIV\text{-}Gag\text{-}eGFP}$ by mDC LPS was completely blocked in the presence of the GM3 polar head group (sialyllactose), while equal concentrations of lactose (lacking the sialic acid group) had no effect (Fig. 41 C). Taken together, these data clearly show that the sialyllactose moiety of gangliosides is the molecular determinant required for efficient VLP and LUV recognition and capture by mDC LPS.

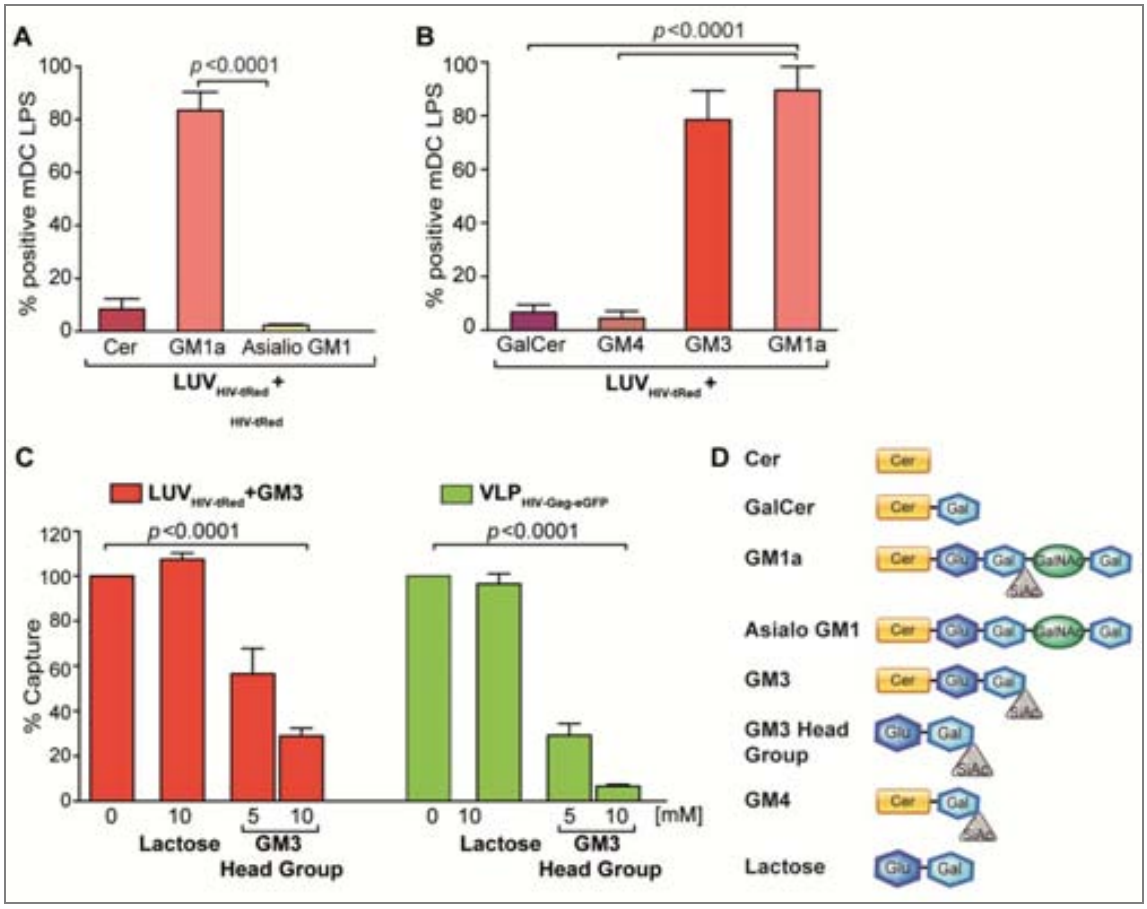


**Figure 41. Identification of the molecular recognition domain present in gangliosides essential for mDC LPS capture. (A)** Comparative mDC LPS capture of distinct $LUV_{HIV\text{-}tRed}$ containing Cer, GM1a, or GM1 lacking sialic acid (Asialo GM1). A total of 2 x $10^5$ mDC LPS were pulsed for 4 hours at 37ºC in 5% $CO_2$ with 100 µM of LUV, washed with PBS, and assessed by FACS to obtain the percentage of tRed-positive cells. mDC LPS capture significantly higher amounts of GM1a-containing $LUV_{HIV\text{-}tRed}$ than Asialo GM1-containing $LUV_{HIV\text{-}tRed}$ ($p$<0.0001, paired t test). **(B)** Comparative mDC LPS capture of distinct $LUV_{HIV\text{-}tRed}$ containing GalCer, GM4, GM3, or GM1a. A total of 2 x $10^5$ mDC LPS were pulsed for 4 hours at 37ºC in 5% $CO_2$ with 100 µM of LUV, washed, and assessed by FACS to obtain the percentage of tRed-positive cells. mDC LPS capture significantly higher amounts of GM1a-containing $LUV_{HIV\text{-}tRed}$ than GalCer or GM4-containing $LUV_{HIV\text{-}tRed}$ ($p$<0.0001, paired t test). **(C)** Graph representing the relative capture of GM3-containing $LUV_{HIV\text{-}tRed}$ and $VLP_{HIV\text{-}Gag\text{-}eGFP}$ by mDC LPS that had been pre-incubated with 10 mM of soluble lactose or with 5–10 mM of GM3 carbohydrate polar head group, normalized to the level of LUV/VLP capture by mock-treated mDC LPS (set at 100%). mDC LPS captured fewer particles upon treatment with GM3 polar head group ($p$<0.0001, paired t test). Data show mean values and SEM from three independent experiments including cells from at least nine donors. **(D)** Schematic view of the molecules present in the LUV used in the assays or reagents employed for these experiments.

## 4. Sialyllactose in membrane gangliosides of HIV-1 is required for viral capture by mDC LPS

To determine whether the results obtained with LUV and VLP also hold true for the authentic virus, we performed experiments with wild-type $HIV_{NL4-3}$ produced in primary T cells. Similar to our previous data [169], a high percentage of mDC LPS captured HIV-1, while uptake into iDC was much less efficient ($p$=0.0047, one sample t test) (Fig. 42 A). To confirm the importance of viral gangliosides for mDC LPS capture, we purified $HIV_{NL4-3}$ from primary $CD4^+$ T cells pre-treated or not with the GSL biosynthesis inhibitor NB-DNJ. The NB-DNJ is a glucose analogue that blocks the first step of GSL biosynthesis by inhibiting of the Cer-specific glycosyltransferase. The NB-DNJ-treatment of producer cells rendered $HIV_{NL4-3}$ deficient in GSL levels. Consistently with the results obtained with LUV and VLP, HIV-1 capture was strongly reduced for virus obtained from NB-DNJ-treated producer cells compared to control virus ($p$<0.0001, one sample t test) (Fig. 42 B). Consequently, alterations in HIV-1 incorporation of GSL lead to a dramatic decrease in virus capture by mDC LPS. To directly determine the importance of the sialyllactose head group for mDC LPS capture of authentic HIV-1, we performed competition experiments of $HIV_{NL4-3}$ in the presence of the GM3 polar head group. The experiments showed a strong reduction of virus capture in the presence of the GM3 polar head group, but not in the presence of lactose, which lacks the sialic acid moiety of the GM3 head gorup ($p$<0.0001, one sample t test) (Fig. 42 C). These data corroborate the observations obtained with liposomes and VLP for authentic HIV-1 from primary $CD4^+$ T cells, and confirm that the presence of the sialic acid moiety in gangliosides is necessary for HIV-1 recognition by mDC LPS.

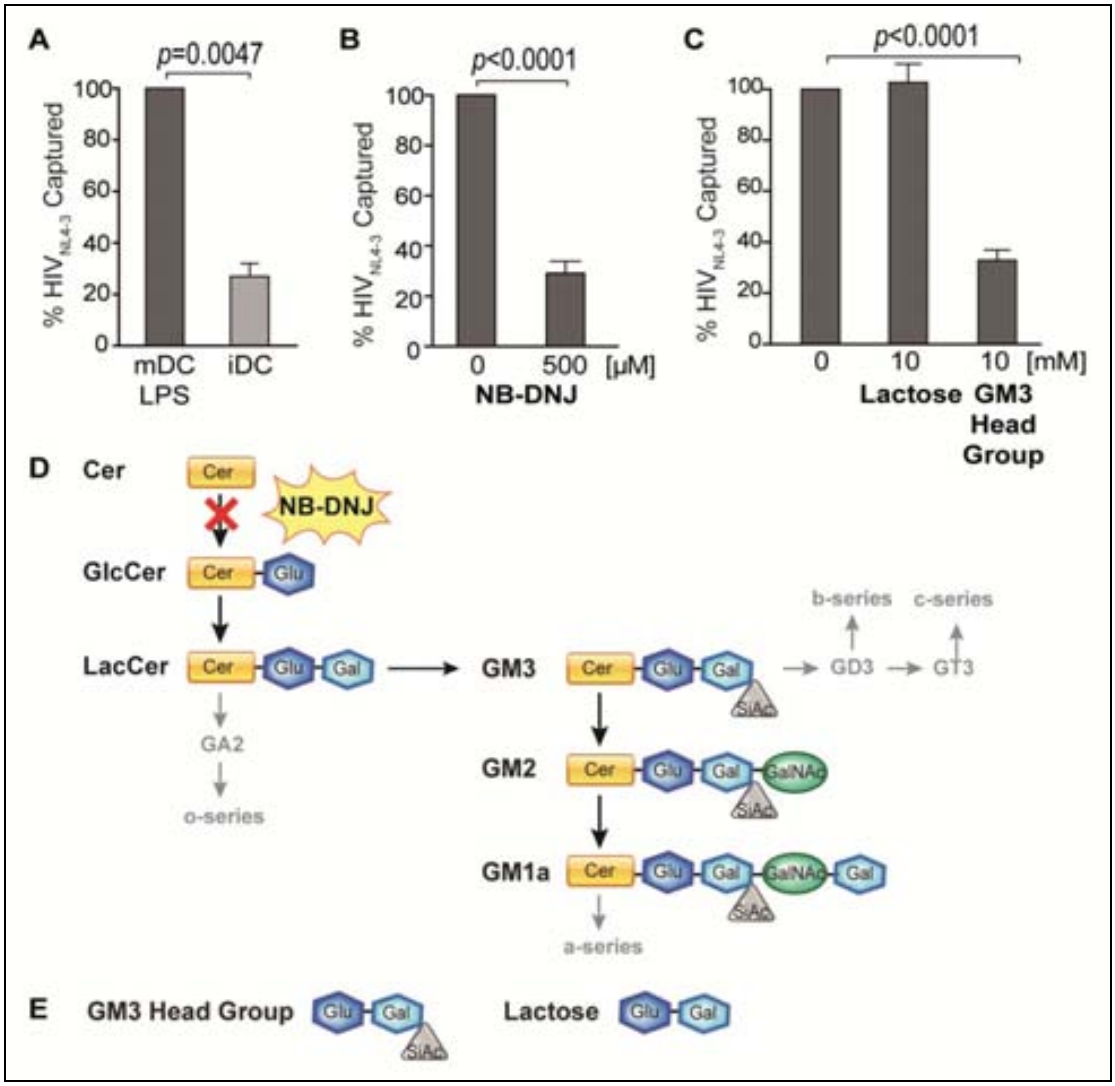


**Figure 42. Sialyllactose in membrane gangliosides is required for HIV-1 capture by mDC LPS. (A)** Relative capture of $HIV_{NL4-3}$ produced in primary cells by mDC LPS and iDC. DC were pulsed for 4 hours at 37ºC in 5% $CO_2$ with equal amounts of a $HIV_{NL4-3}$ produced in stimulated PBMC, extensively washed, and then assayed for cell-associated $p24^{Gag}$ content by ELISA. Results are expressed as the percentage of $HIV_{NL4-3}$ captured by iDC relative to mDC LPS, normalized to 100% of viral capture per blood donor. Viral uptake was increased in mDC LPS compared to iDC ($p$=0.0047, one sample t test). Data show mean values and SEM from two independent experiments including cells from three donors. **(B)** Relative capture of $HIV_{NL4-3}$ produced in $CD4^+$ T cells that had been treated or not with the GSL inhibitor NB-DNJ. mDC LPS were pulsed for 4 hours at 37ºC in 5% $CO_2$ with equal amounts of $HIV_{NL4-3}$ generated in NB-DNJ-treated or mock-treated $CD4^+$ T cells and then assayed for $p24^{Gag}$ content by ELISA. mDC LPS captured less $HIV_{NL4-3}$ generated in NB-DNJ-treated $CD4^+$ T cells ($p$<0.0001, one sample t test). Data show mean values and SEM from two independent experiments including cells from six donors. **(C)** Competition of $HIV_{NL4-3}$ capture by mDC LPS in the presence of the GM3 head group. mDC LPS were either pre-incubated with 10 mM lactose, 10 mM GM3 head group, or mock treated, and were then pulsed for 4 hours at 37ºC in 5% $CO_2$ with equal amounts of $HIV_{NL4-3}$ generated in $CD4^+$ T cells. Virus uptake was assayed by ELISA for $p24^{Gag}$. $HIV_{NL4-3}$ capture was strongly reduced upon pre-treatment with the GM3 head group, but not lactose ($p$<0.0001, one sample t test). Data show mean values and SEM from two independent experiments including cells from six donors. **(D)** Diagram showing the step were NB-DNJ inhibits the GSL synthesis. NB-DNJ blocks the addition of a glucose molecule to Cer to form glucosulceramide (GlcCer). **(E)** Schematic view of the molecules GM3 head group and lactose used in the viral capture experiments.

## 5. Sialyllactose in membrane gangliosides of HIV-1 is required for mDC LPS-mediated HIV-1 *trans*-infection

HIV-1 capture by mDC LPS has been shown to promote *trans*-infection of $CD4^+$ T cells and other target cells [145, 169, 173, 177, 186], and we therefore analyzed whether sialyllactose recognition by mDC LPS is also important for viral transmission. Similarly to viral capture assays illustrated in Fig. 42, mDC LPS-mediated *trans*-infection experiments were performed with GSL-deficient $HIV_{NL4-3}$ produced by NB-DNJ-treated $CD4^+$ T cells or in the presence of the GM3 polar head group. Co-culturing mDC LPS that had been exposed to an equivalent amount of infectious $HIV_{NL4-3}$ derived from NB-DNJ-treated or untreated primary $CD4^+$ T cells with the TZM-bl reporter cell line revealed a strong reduction of *trans*-infection for the virus from inhibitor-treated cells compared to control virus ($p$=0.0404, paired t test) (Fig. 43 A). We also observed a strong reduction of *trans*-infection for mDC LPS pulsed with $HIV_{NL4-3}$ in the presence of the GM3 polar head group and subsequently incubated with TZM-bl cells (Fig. 43 B) ($p$=0.0197, paired t test). These results were further confirmed when we co-cultured HIV-1–pulsed mDC LPS with activated primary $CD4^+$ T cells. Co-cultures were performed in the presence or absence of the protease inhibitor SQV to distinguish net *trans*-infection from re-infection events (Fig. 43 C and D; *left* and *right panels*, respectively). Infection of primary $CD4^+$ T cells was strongly enhanced when they were co-cultured with HIV-1 pulsed mDC LPS (Fig. 43 C and D; filled bars). This effect was abrogated when mDC LPS were pulsed with virus produced from NB-DNJ-treated cells (Fig. 43 C) or cultured with the GM3 polar head group (Fig. 43 D). Consequently, these data indicate that the sialyllactose moiety of gangliosides is the molecular determinant required for efficient HIV-1 capture by mDC LPS and for subsequent viral *trans*-infection of target cells.

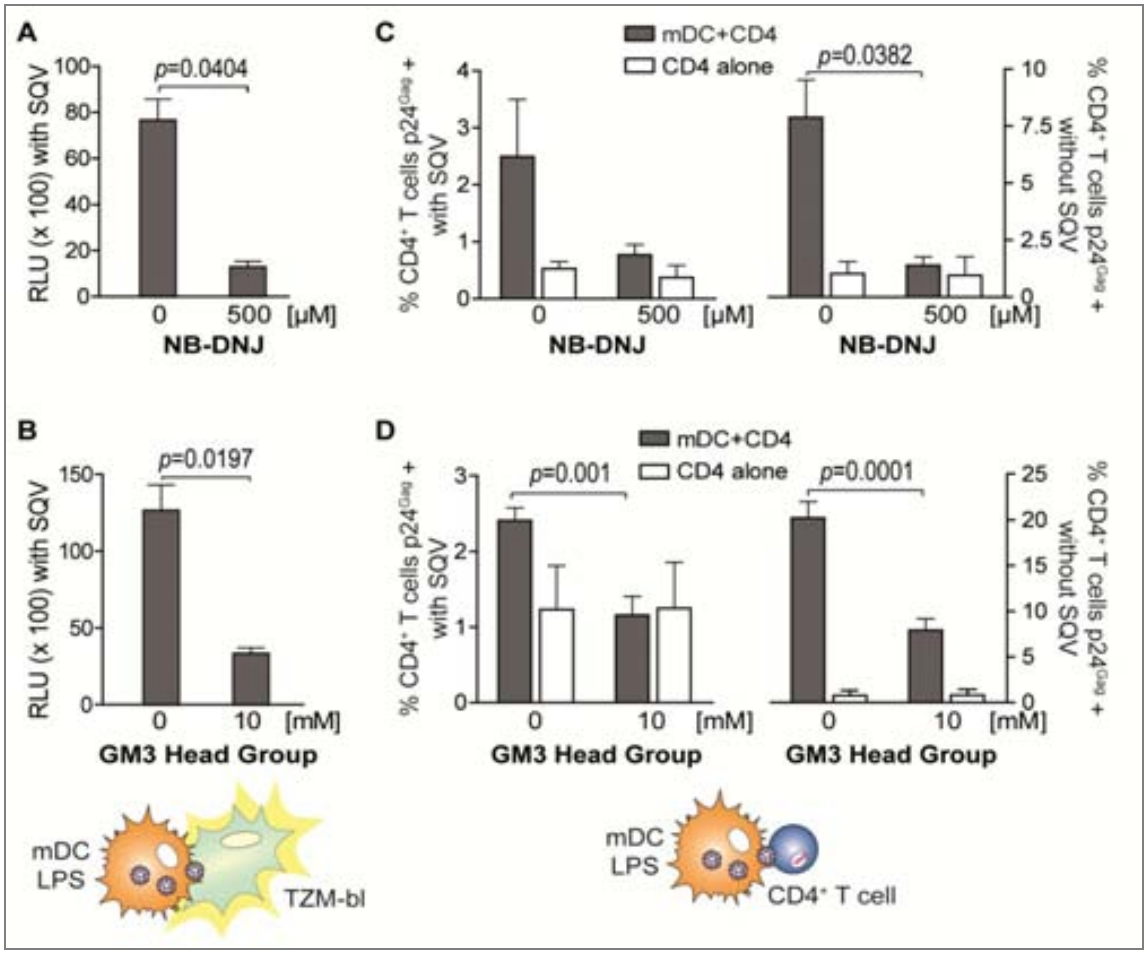


**Figure 43. Sialyllactose in membrane gangliosides is required for mDC LPS *trans*-infection of HIV-1. (A)** Transmission of $HIV_{NL4-3}$ produced in NB-DNJ or mock-treated $CD4^+$ T cells to the TZM-bl target cell line. mDC LPS treated as described in Fig. 42 B but pulsed with an equal MOI of 0.1 were extensively washed and co-cultured with TZM-bl cells in the presence of SQV (to prevent second round infection) for 48 hours before measurement of luciferase activity. *Trans*-infection was less efficient for $HIV_{NL4-3}$ generated in NB-DNJ-treated compared to untreated $CD4^+$ T cells ($p$=0.0404, paired t test). Data show mean values and SEM from one experiment including cells from three donors. **(B)** Competition of *trans*-infection of $HIV_{NL4-3}$ in the presence of the GM3 head group. mDC LPS treated as described in Fig. 42 C but pulsed with an MOI of 0.1 were extensively washed and co-cultured with TZM-bl in the presence of SQV for 48 hours before measurement of luciferase activity. *Trans*-infection was significantly reduced by the GM3 head group ($p$=0.0197, paired t test). Data show mean values and SEM from two independent experiments including cells from six donors. **(C)** *Trans*-infection of activated primary $CD4^+$ T cells by co-culture with mDC LPS pulsed with $HIV_{NL4-3}$ produced in NB-DNJ or mock-treated $CD4^+$ T cells. mDC LPS were pulsed with $HIV_{NL4-3}$ at an equivalent MOI of 0.1 as described in Fig. 42 B. Unwashed pulsed mDC LPS were subsequently co-cultured with primary $CD4^+$ T cells for 48 hours before measuring the intracellular $p24^{Gag}$ content in the lymphocyte gate (CD2+ and CD11c- cells) by FACS (filled bars). Equivalent amounts of cell-free $HIV_{NL4-3}$ were used as a control (open bars). Experiments were done in the presence (left panel) or absence (right panel) of SQV to distinguish net *trans*-infection from re-infection events, respectively. mDC LPS transferred less $HIV_{NL4-3}$ generated in NB-DNJ-treated $CD4^+$ T cells ($p$=0.0382, paired t test). Data show mean values and SEM from one representative experiment including cells from three donors. **(D)** *Trans*-infection of activated primary $CD4^+$ T cells by co-culture with mDC LPS pulsed with $HIV_{NL4-3}$ in the presence or absence of the GM3 head group. mDC LPS were pulsed with $HIV_{NL4-3}$ at an equivalent MOI of 0.1 as described in Fig. 42 C. Unwashed pulsed mDC LPS were subsequently co-cultured with primary $CD4^+$ T cells for 48 hours before measuring the intracellular $p24^{Gag}$ content in the lymphocyte gate (CD2+ and

CD11c- cells) by FACS, as detailed in (H). mDC LPS transferred less $HIV_{NL4-3}$ upon treatment with the GM3 head group (P-values on the graph, paired t test). Data show mean values and SEM from two independent experiments including cells from six donors.

# Chapter 7

## RESULTS IV

*Siglec-1 is a novel DC receptor that mediates HIV-1 capture and* trans*-infection through recognition of viral membrane gangliosides*

*This chapter is part of the manuscript:*

Izquierdo-Useros N., Lorizate M., Puertas M.C., **Rodriguez-Plata M.T.**, Zangger N., Erikson E., Pino M., Erkizia I., Glass B., Clotet B., Keppler O.T., Telenti A., Kräusslich H.G., Martinez-Picado J. **Siglec-1 is a novel dendritic cell receptor that mediates HIV-1 *trans*-infection through recognition of viral membrane gangliosides.** *PLoS Biology*. 2012;10(12):e1001448. *Second authorship*.

Besides being crucial for the induction of antiviral immunity, DC play an essential role in HIV-1 pathogenesis. DC can capture HIV-1 and mediate *trans*-infection, an ability that is markedly enhanced upon maturation with certain stimuli, such as LPS [169, 173, 177, 186]. DC-SIGN and other CLR have been identified as binding factors for HIV-1 but do not explain why mDC LPS capture of HIV-1 is independent of viral glycoproteins [169]. Instead, HIV-1 capture is markedly sensitive to reductions in viral GSL content [172] and relies on HIV-1 incorporation of membrane gangliosides [see Results Chapter 6 – Results III and [290, 291]]. Sialyllactose was finally identified as the molecular recognition pattern in HIV-1 that mediates the gp120-independent capture by DC [*see* Chapter 6 – Results III, and [290]]. However, the mDC LPS attachment factor responsible for this HIV-1 Env-independent binding remains uncharacterized.



In this study we identified the sialic acid-binding Ig-like lectin 1 (Siglec-1, CD169) as the surface receptor on mDC LPS that boosts their gp120-independent uptake of HIV-1 and their capacity to *trans*-infect $CD4^+$ T cells. Siglec-1 captures HIV-1 through its interaction with sialyllactose-containing gangliosides exposed on the viral membrane, but it can also function as a general binding molecule for vesicle carrying sialyllactose in their membrane, including other viruses, exosomes and liposomes. Siglec-1 is essential for HIV-1 capture and subsequent *trans*-infection by mDC LPS, suggesting a key role of Siglec-1 HIV-1 dissemination in activated tissues.

## 1. Siglec-1 is upregulated in highly *trans*-infecting mDC LPS

We and other have shown that sialyllactose on HIV-1 membrane gangliosides is the viral attachment factor for mDC LPS in a gp120-independent manner [*see* Chapter 6 - Results III, and [290, 291]]. To identify the molecule on DC that mediates HIV-1 capture, we performed transcriptome analysis on differentially matured DC with a highly divergent capacity to capture and transmit HIV-1. We used efficiently *trans*-infecting mDC LPS compared them to DC matured in the presence of the clinical grade cocktail ITIP (mDC ITIP), which, as we previously observed [*see* Chapter 4 - Results I, and [273]], exhibit dramatic reduced HIV-1 capture and *trans*-infection capacity (Fig. 44 A, paired t test).



We focused our analysis on the Siglec family (including CD83) because these type I transmembrane proteins have an amino-terminal V-set domain that had been shown to interact with sialylated ligands [292]. Most members of the family were equally expressed in mDC LPS and mDC ITIP, and this was also observed for the maturation marker CD86 (Fig. 44 B). *DC-SIGN*, *SIGLEC7*, and *SIGLEC14* were slightly upregulated in mDC LPS, but this difference was not statistically significant for *DC-SIGN* and marginally significant for *SIGLEC14* and *SIGLEC7*, respectively ($p$=0.03 and $p$=0.04). In contrast, *SIGLEC1* expression was strongly upregulated in mDC LPS compared to mDC ITIP with genome-wide significance ($p$=3.5x10$^{-4}$; Fig. 44 B). Furthermore, *SIGLEC1* ranked 20$^{th}$ of all differentially regulated genes in comparative transcriptome analysis. The differential expression of Siglec-1 in mDC LPS and ITIP was confirmed by quantitative real-time PCR (qRT-PCR; Fig. 44 C) and flow cytometry (Fig. 44 D and E). Comparison with iDC also revealed a significantly higher expression level and surface density of Siglec-1 in mDC LPS (Fig. 44 D and E).

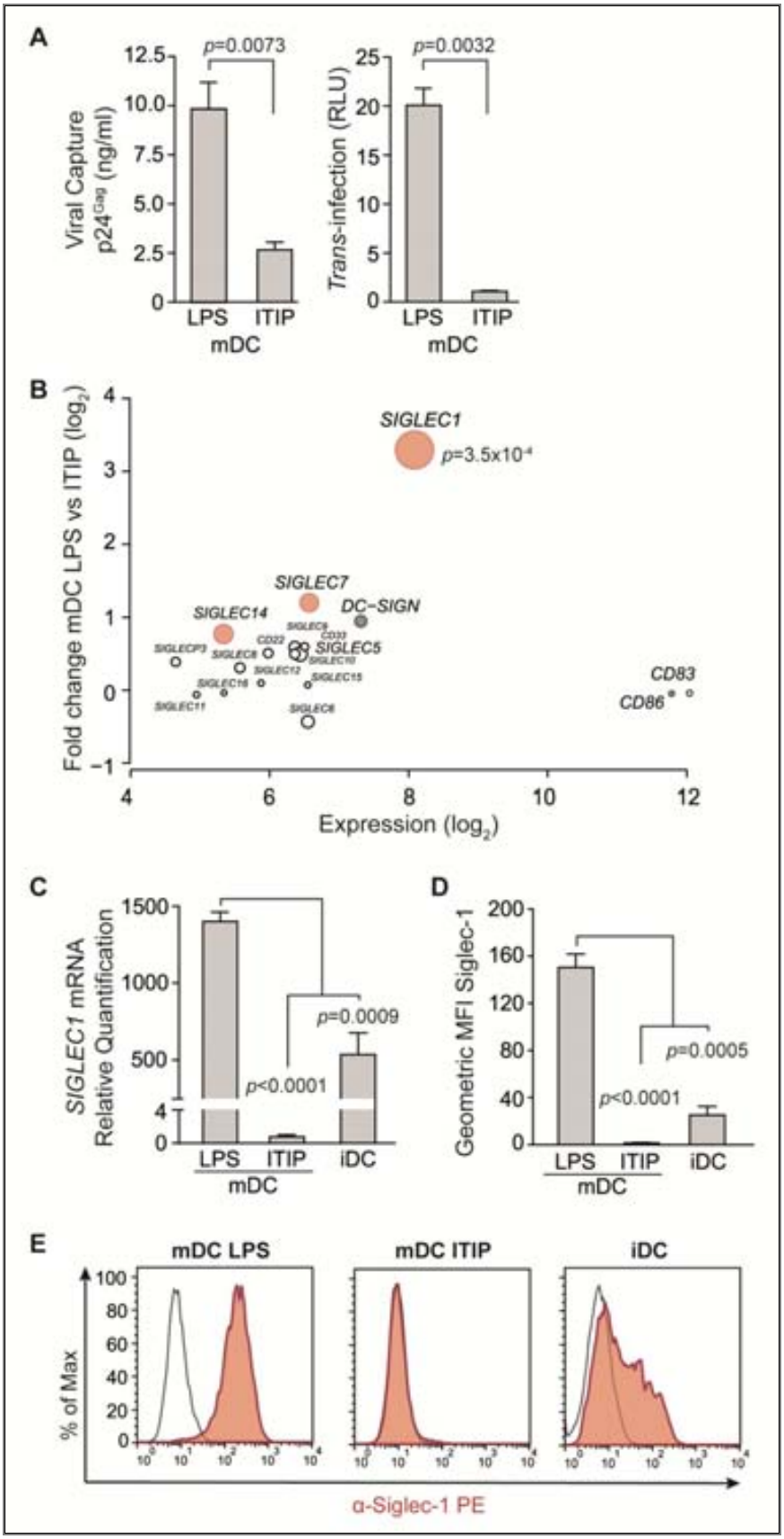


**Figure 44. Siglec-1 is upregulated in highly *trans*-infecting mDC LPS. (A)** *Left panel,* Comparative HIV-1 capture of mDC LPS and mDC ITIP: cells were cultured with $HIV_{NL4-3}$ from

HEK-293T cells for 6 hours at 37ºC in 5% $CO_2$, washed, and lysed to measure viral $p24^{Gag}$ antigen by ELISA. *Right panel,* Comparative transmission of captured $HIV_{NL4-3}$ from mDC LPS and mDC ITIP to the reporter cell line TZM-bl (CD4+). Graphs show mean values and SEM from two independent experiments including cells from six donors. **(B)** Plot of *SIGLEC* genes (in open circles), *CD86* and *DC-SIGN* (in grey circles) computing the fold change in mDC LPS compared to mDC ITIP, and the average gene expression across all samples. Circle size is inversely proportional to adjusted *p* values. Highlighted in red are statistically differentially expressed genes. Analysis was performed with DC from four donors matured in parallel with the different stimuli. **(C)** Relative quantification of *SIGLEC1* mRNA expression levels in mDC LPS, mDC ITIP and iDC analyzed by qRT-PCR. Measurements were normalized using the endogenous control housekeeping gene *Beta Glucuronidase*. Data show means and SEM of samples from six donors. **(D)** Cell surface expression of Siglec-1 in mDC LPS, mDC ITIP and iDC analyzed by flow cytometry with mAb 7–239-PE. Geometric mean fluorescence intensity (MFI) of Siglec-1. **(E)** Representative profiles of Siglec-1 staining in mDC LPS, mDC ITIP and iDC derived from one donor.



## 2. Siglec-1 expressed in mDC LPS capture distinct ganglioside containing vesicles, such as HIV-1 viral-like particles and liposomes

To test whether Siglec-1 is the surface molecule on mDC LPS responsible for the capture of vesicles and viruses that carry sialyllactose-containing gangliosides in the outer leaflet of their membrane, we used a previously established flow cytometry assay [*see* Chapter 6 – Results III and [172, 290]]. This assay makes use of HIV-1 virus-like particles lacking the viral envelope glycoproteins and carrying a fusion of the viral structural protein Gag with eGFP ($VLP_{HIV\text{-}Gag\text{-}eGFP}$). These fluorescent VLP follow the same trafficking route as wild-type HIV-1 in mDC LPS [172]. VLP capture of mDC LPS was evaluated in the presence of mAb against different Siglecs or mannan, a C-type lectin inhibitor blocking the HIV-1 interaction with DC-SIGN. Besides Siglec-1, we included mAb against CD83, highly expressed in mDC ITIP and mDC LPS (Fig. 44 B); Siglec-7, moderately upregulated in mDC LPS (Fig. 44 B); and Siglec- 5/14 too, due to their high homology to the V-set domain of Siglec-1. VLP capture was almost completely abolished when mDC LPS were pre-treated with the α-Siglec-1 mAb 7D2 ($p<0.0001$, Fig. 45 A). However, pretreatment with mAb against other Siglec family members or blockade of DC-SIGN with mannan had no effect (Fig. 45 A). We have previously shown that Texas Red (tRed) labeled Large Unilamellar Vesicles (LUV) mimicking the size and lipid composition of HIV-1 and containing the ganglioside GM1 ($LUV_{HIV\text{-}tRed}$) follow the same trafficking route as $VLP_{HIV\text{-}GageGFP}$ in mDC LPS. Thus, binding and capture in both cases depends on the recognition of sialyllactose exposed in gangliosides of the vesicle membrane [*see* Chapter 6 – Results III and [290]].

Accordingly, capture of GM1-containing $LUV_{HIV\text{-}tRed}$ by mDC LPS was efficiently and specifically inhibited by the α-Siglec-1 mAb 7D2 ($p$<0.0001; Fig. 45 B). The residual capture by α-Siglec-1-treated mDC LPS was similar to that exhibited by untreated mDC LPS capturing $LUV_{HIV\text{-}tRed}$ containing GM1 without the sialic acid group (Asialo GM1), confirming that sialic acid in the vesicle membrane is crucial for Siglec-1 recognition ($p$<0.0001; Fig. 45 B). Specificity of the α-Siglec-1 mAb 7D2–mediated inhibition was confirmed by pre-incubation of this mAb with different Siglec proteins. Pre-incubation with purified Siglec-1 completely restored VLP capture, while pre-incubation with purified Siglec-7, -5/14, or CD83 had no effect (Fig. 45 C). Consequently, this specific inhibition of sialyllactose-dependent mDC LPS capture of VLP and LUV identified Siglec-1 as the relevant recognition receptor.

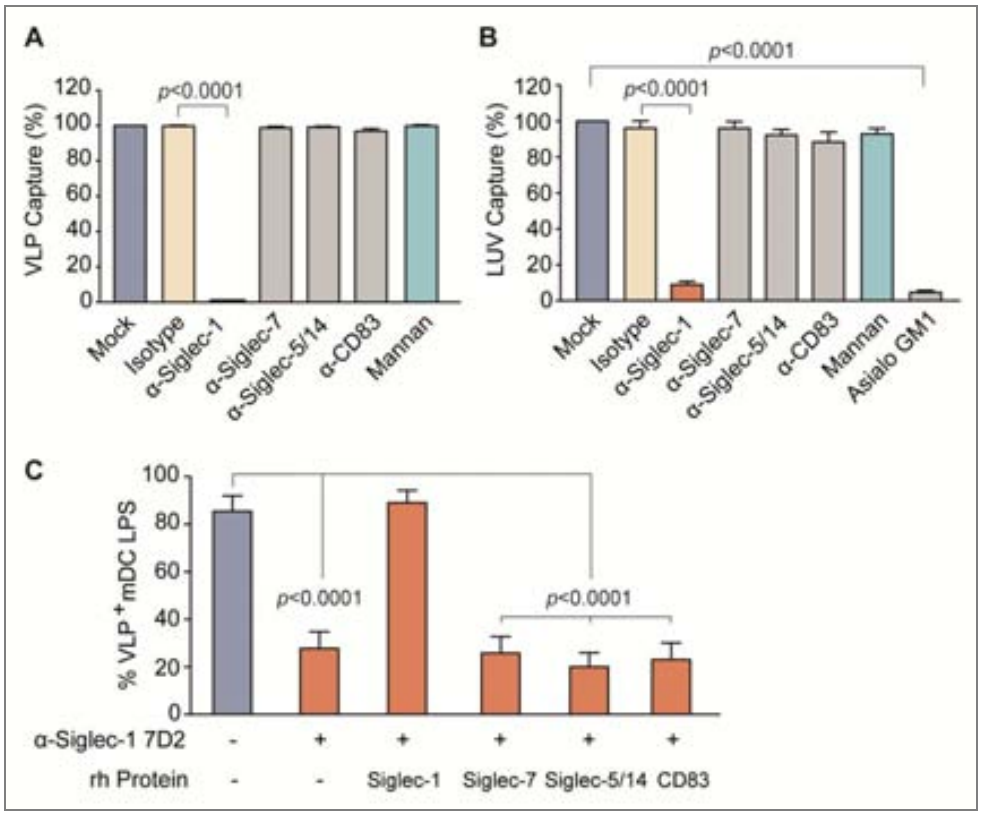


**Figure 45. Siglec-1 expressed in mDC LPS capture distinct ganglioside containing vesicles, such as HIV-1 viral-like particles and liposomes. (A)** Relative capture of $VLP_{HIV\text{-}Gag\text{-}eGFP}$ by mDC LPS that had been pre-incubated with 10 µg/ml of the indicated mAb or 500 µg/ml of mannan before VLP exposure for 30 minutes at 37ºC in 5% $CO_2$. Values are normalized to the level of VLP capture by mock-treated mDC LPS (set at 100%). Data show mean values and SEMs from three experiments including cells from nine donors. **(B)** Relative capture of GM1 containing $LUV_{HIV\text{-}tRed}$ by mDC LPS as described in (A). Data show mean values and SEM from two experiments including cells from six donors. **(C)** Capture of $VLP_{HIV\text{-}Gag\text{-}eGFP}$ by mDC LPS that had been pre-incubated with or without 2 µg/ml of α-Siglec-1 mAb 7D2 previously treated or not with at least a 100-fold molar excess of the indicated human recombinant proteins. Of note, Siglec-14 shares 100% of amino acid homology with Siglec-5 in the V-set domain. Data show mean values and SEMs from three experiments including cells from nine donors.

If Siglec-1 serves as a recognition receptor on DC, its surface expression should correlate with their respective VLP capture ability. Capture was low in iDC and stable over time (Fig. 46 A, *left graph*), while VLP capture was strongly enhanced following LPS treatment (Fig. 46 A, *right graph*). This increased VLP capture ability directly correlated with a strong upregulation of Siglec-1 surface expression on mDC LPS (Fig. 46 A, *right graph*). We also performed quantitative flow cytometry analysis to determine the absolute number of Siglec-1 Ab Binding Sites (ABS) on mDC LPS, mDC ITIP and iDC (Fig. 46 B). The VLP capture capacity of these distinct DC subtypes was strongly correlated with the mean number of Siglec-1 ABS expressed per cell (Fig. 46 B; ρ=0.9695). Altogether, these experiments show a direct correlation between Siglec-1 expression on the DC surface and their respective VLP capture capacity.



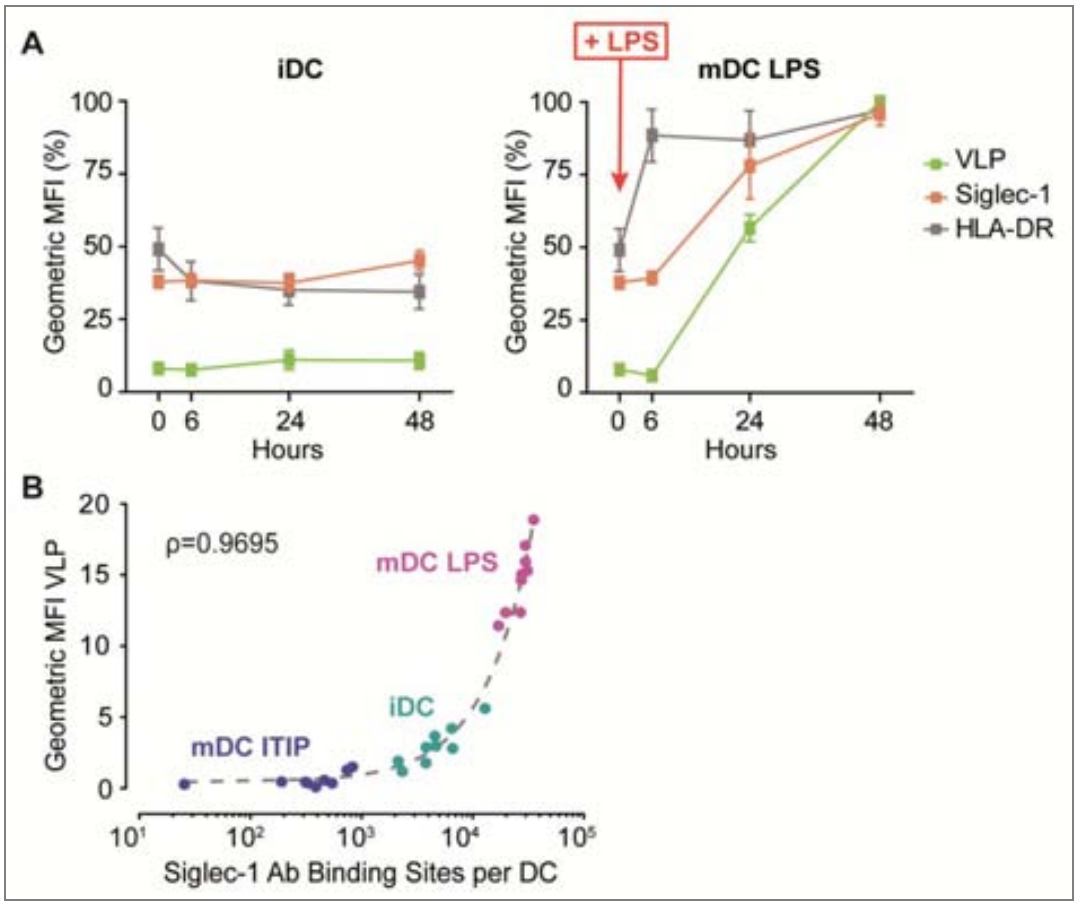


**Figure 46. (A)** Kinetics of $VLP_{HIV\text{-}Gag\text{-}eGFP}$ capture by iDC *(left graph)* and mDC LPS *(right graph)* compared to the expression of Siglec-1 over time, assessed after LPS addition to mDC. Cells were pulsed for 1 hour at 37ºC in 5% $CO_2$ with $VLP_{HIV\text{-}Gag\text{-}eGFP}$ and labeled for Siglec-1 and HLA-DR in parallel at the indicated time points. For comparative purposes, the maximum geometric MFI values obtained by flow cytometry for each donor were set at 100%. Data show mean values and SEM including cells from three donors. **(B)** Positive correlation (ρ=0.9695) between the geometric MFI of captured VLP and the mean number of Siglec-1 Ab Binding Sites per cell in mDC LPS, mDC ITIP and iDC. Data show values from three experiments including cells from nine donors.

### 3. Siglec-1 is the HIV-1–binding surface protein that mediates HIV-1 capture in mDC LPS

To extend these observations to authentic virus and determine that Siglec-1 is the surface molecule on mDC LPS responsible for the capture of HIV-1, we performed blocking experiments with infectious virus. To that end, DC were pre-incubated pre-incubated with the α-Siglec-1 mAb 7D2 or mannan, a C-type lectin inhibitor, and then exposed to $HIV_{NL4-3}$ to determine the contribution of the different molecules to DC viral capture (Fig. 47 A). Again, mDC LPS captured significantly more $HIV_{NL4-3}$ than iDC or mDC ITIP (Fig. 47 B). Notably, the α-Siglec-1 mAb 7D2 inhibited HIV-1 capture of mDC LPS by 80%, representing a 3.7-fold decrease ($p$=0.0019; Fig. 47 B), while pre-treatment with mannan had no effect. Similarly, pre-treatment of iDC with the α-Siglec-1 mAb 7D2 reduced HIV-1 capture by 60% ($p$=0.0005; Fig. 47 B), indicating that even at lower surface expression levels of Siglec-1 on iDC (Fig. 44 C, D and E), this receptor still constitutes an important capture moiety. Consistently, capture inhibition by α-Siglec-1 mAb 7D2 was much weaker on mDC ITIP ($p$=0.0010, Fig. 47 B), which exhibited the lowest Siglec-1 surface expression (Fig. 44). However, viral uptake on mDC ITIP was also slightly inhibited by mannan ($p$=0.0116; Fig. 47 B), suggesting a role of C-type lectins on HIV-1 capture by this DC subtype. Furthermore, the effect of the α-Siglec-1 mAb 7D2 on HIV-1 capture was dependent on blocking cell surface Siglec-1, as addition of the inhibitor after virus exposure had no effect (Fig. 47 C). These results strongly suggest that Siglec-1 is the molecule responsible for HIV-1 capture by DC, especially upon triggering of Siglec-1 expression by LPS.

Next, we investigated whether Siglec-1 traffics together with sialylated ligands, such as ganglioside-containing liposomes ($LUV_{HIV-tRed}$), VLP, or HIV-1, reaching the same sac-like compartment where these particles are stored [*see* Chapter 6 – Results III and [172, 290]]. mDC LPS were pulsed with these different fluorescent particles and subsequently stained with the α-Siglec-1 Alexa 488 mAb 7–239 (Fig. 48). Confocal microscopy revealed extensive co-localization of Siglec-1 with $HIV\text{-}1_{Cherry}$, $VLP_{HIV\text{-}Gag\text{-}Cherry}$, and GM1-containing $LUV_{HIV-tRed}$, and in the same compartment (Fig. 48 A, B and C, respectively).

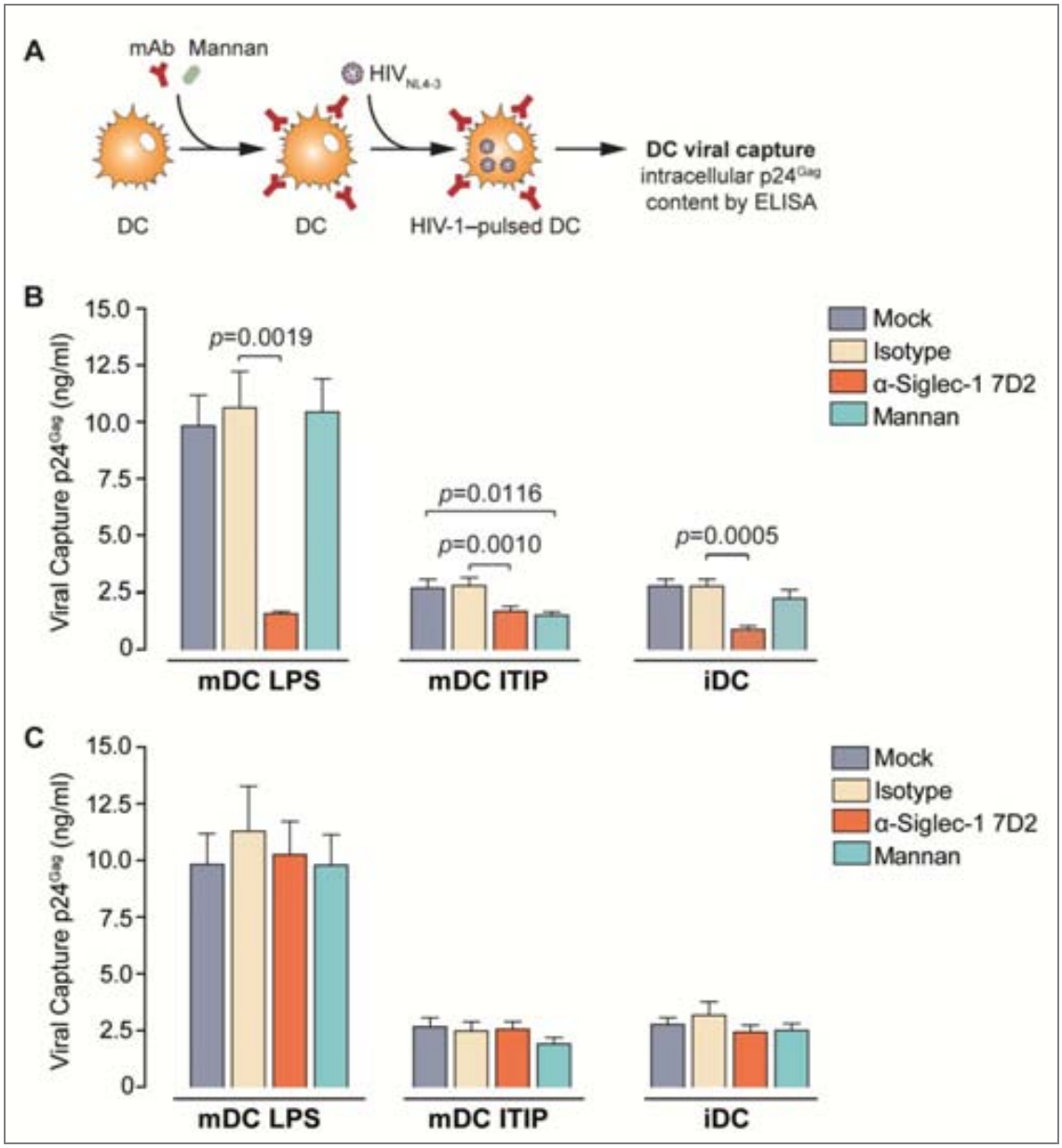


**Figure 47. Siglec-1 is the HIV-1–binding surface protein that mediates HIV-1 capture in mDC LPS. (A)** Experimental procedure assess the relevance of Siglec-1 in HIV-1 uptake by DC. DC were pre-incubated with 10 µg/ml of the indicated mAb or 500 µg/ml of mannan for 30 minutes at 16ºC, then expose to $HIV_{NL4-3}$ for 5 hours at 37ºC in 5% $CO_2$. Cells were washed, and lysed to measure $p24^{Gag}$ by ELISA. **(B)** Comparative HIV-1 capture by mDC LPS, mDC ITIP and iDC as described in panel A. Data show mean values and SEM from two experiments including cells from six donors. **(C)** Comparative capture of HIV-1 by mDC LPS, mDC ITIP and iDC first exposed to the $HIV_{NL4-3}$ for 5 hours at 37ºC in 5% $CO_2$ and then treated with the indicated reagents for 30 minutes before washing. Cells were then lysed and assessed by $p24^{Gag}$ ELISA. Data show mean values and SEM from two experiments including cells from six donors. Statistical analysis: paired t test.

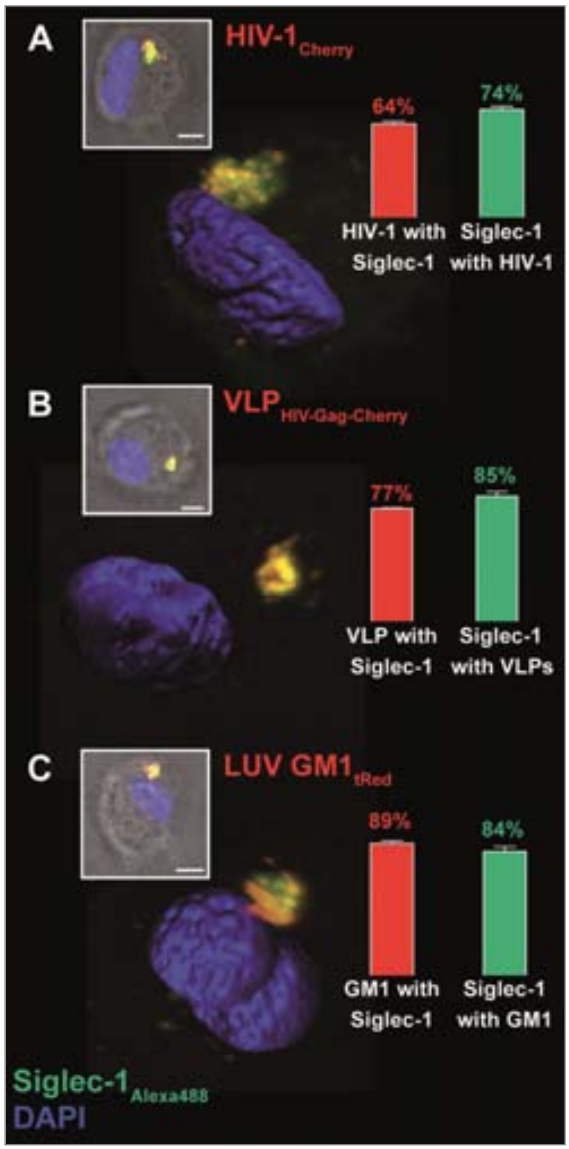


**Figure 48. Siglec-1 traffics with HIV-1 to the same sac-like compartment in mDC LPS.** Confocal microscopy analysis of mDC LPS pulsed for 4 hours with **(A)** HIV-$1_{Cherry}$, **(B)** $VLP_{HIV\text{-}Gag\text{-}Cherry}$ or **(C)** GM1-containing $LUV_{HIV\text{-}tRed}$, fixed, permeabilized, and then stained for Siglec-1 with mAb 7–239-Alexa 488. *Inset*, Merge of the bright field and maximun fluorescence intensity (scale bar: 5 μm). *3D images*, Isosurface representation of DAPI stained nucleus and maximum fluorescence intensity of the sac-like compartment where particles and Siglec-1 accumulate are shown in a 3D volumetric x-y-z data field. *Bar graphs*, Quantification of the percentage of HIV-$1_{Cherry}$, $VLP_{HIV\text{-}Gag\text{-}Cherry}$ or GM1-containing $LUV_{HIV\text{-}tRed}$ co-localizing with Siglec-1-Alexa 488 7–239 and vice versa, obtained analyzing at least 50 compartments from mDC LPS of two donors. The mean and standard deviation of the thresholded correlation coefficient of Pearson (obtained considering all the images) were 0.77±0.07, indicating co-localization.

## 4. HIV-1 capture by Siglec-1 facilitates mDC LPS-mediated HIV-1 *trans*-infection

To assess the relevance of Siglec-1 for HIV-1 *trans*-infection, we pulsed mDC LPS, mDC ITIP and iDC with equal amounts of infectious virus in the presence or absence of blocking reagents, as in Fig. 47 A and B, and co-cultured them with the $CD4^+$ reporter cell line TZM-bl (Fig. 49 A). Controls performed with the protease inhibitor SQV, which abolishes production of infectious virus, demonstrated that this assay measured only *trans*-infection of reporter cells by DC-captured virus without a contribution from potentially de novo infected DC (Fig. 49 B and C, last bars).

Pretreatment of mDC LPS with the α-Siglec-1 mAb 7D2 before viral capture inhibited HIV-1 *trans*-infection by 85%, which represents an 18.6-fold decrease (*p*=0.0052; Fig. 49 B). On the contrary, blocking of C-type lectins such as DC-SIGN through mannan had a slight impact on mDC LPS-mediated HIV-1 *trans*-infection (*p*=0.0369; Fig. 49 B) despite having no effect on viral capture (Fig. 47 A). Analogously, pretreatment of iDC with α-Siglec-1 mAb 7D2 reduced HIV-1 *trans*-infection by 55%, a 2.7-fold drop (*p*=0.0091; Fig. 49 B), while pretreatment with mannan induced a minor inhibition (*p*=0.0142; Fig. 49 B). In contrast to mDC LPS and iDC, mDC ITIP-mediated *trans*-infection was not affected by α-Siglec-1 mAb 7D2 but was only blocked by mannan (*p*=0.0014; Fig. 49 B). Addition of any of the inhibitors tested after DC viral pulse had no significant effect on *trans*-infection (Fig. 49 C), except for the α-Siglec-1 mAb 7D2 in mDC LPS (*p*=0.0069). This latter inhibitory effect could not be explained by differences in viral capture (Fig. 47 B) but is most likely attributed to the cell-to-cell adhesion function of Siglec-1 [293], where establishment of infectious synapses might be partially impaired when Siglec-1 is blocked in mDC LPS.



The ambiguous effect of mannan in mDC LPS and iDC, not affecting viral capture but inhibiting HIV-1 *trans*-infection to some extent, could be explained by several hypothesis. First, Siglec-1 can recognize thousand of sialyllactose containing gangliosides in the HIV-1 membrane, which is clearly superior to the interaction of DC-SIGN with only 14±7 envelope trimers per virion [294]. Second, Siglec-1 viral binding via sialyllactose recognition does not discriminate between infectious or non-infectious HIV-1 particles. Therefore, the greater the expression of Siglec-1, the greater the amount of virions captured and transmitted by DC, thus diminishing the relative contribution of DC-SIGN gp120-mediated viral capture to *trans*-infection. Finally, DC-SIGN-mediated viral uptake promotes MHC-I and MHC-II presentation of HIV-1–derived antigens [104, 165], indicating degradation of captured virions and, consequently, reducing the effective viral content in DC to be *trans*-infected.

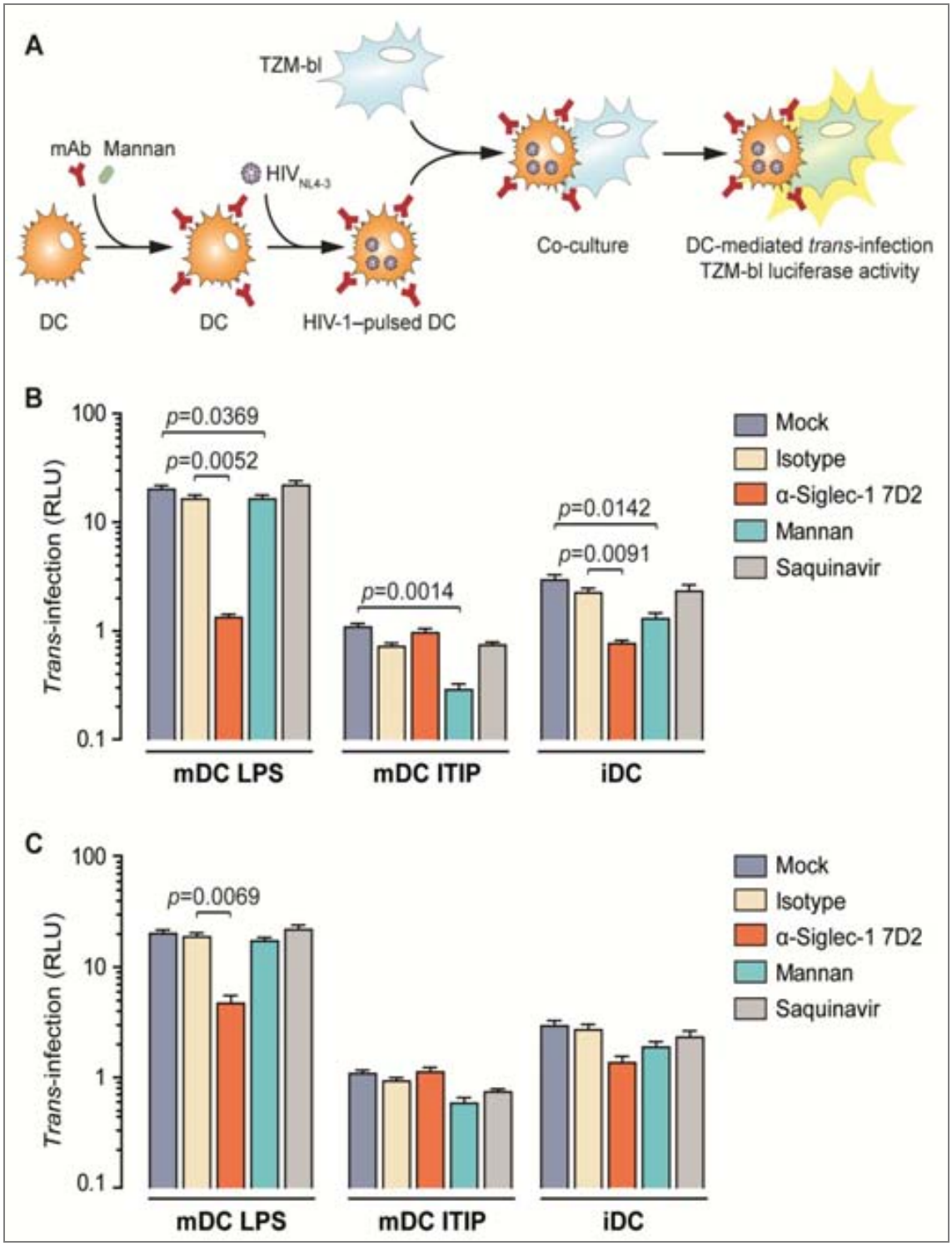


**Figure 49. Siglec-1 mediates HIV-1 *trans*-infection to target cells. (A)** Experimental procedure assess the relevance of Siglec-1 for DC-mediated HIV-1 *trans*-infection. DC were pre-incubated as in Fig. 47 A with 10 µg/ml of the indicated mAb or 500 µg/ml of mannan for 30 minutes at 16ºC, then expose to $HIV_{NL4-3}$ for 5 hours at 37ºC in 5% $CO_2$, and finally washed, and co-cultured with the $CD4^+$ reporter cell line TZM-bl for 48 hours. HIV-1 infection of reporter cells was determined by induced luciferase activity in relative light units (RLU). **(B)** HIV-1 transmission to the $CD4^+$ reporter cell line TZM-bl from mDC LPS, mDC ITIP or iDC treated as described in panel A. One co-culture condition was performed in the presence of the protease inhibitor SQV to distinguish net *trans*-infection from re-infection events. Data show mean values and SEMs from two experiments including cells from six donors. **(C)** HIV-1 transmission from

mDC LPS, mDC ITIP or iDC first exposed to $HIV_{NL4-3}$ and then treated with the indicated reagents for 30 minutes before washing and co-culture with reporter cells. One co-culture condition was performed in the presence of the protease inhibitor SQV to distinguish net *trans*-infection from re-infection events. Data show mean values and SEMs from two experiments including cells from six donors. Statistical analysis: paired t test.

## 5. *SIGLEC1* silencing blocks mDC LPS-mediated HIV-1 capture and *trans*-infection

To verify the essential role of Siglec-1 during HIV-1 capture and *trans*-infection, we applied RNA interference to reduce Siglec-1 expression levels in mDC LPS. To that end, DC were transduced with lentiviral particles coding for different short hairpin RNA (shRNA), which are able to neutralize and degrade the mRNA that has a complementary sequence, thus leading to target gene silencing. Concomitantly, DC were co-infected with vpx-expressing lentiviruses to counteract the restriction factor SAMHD1 and facilitate DC productive infection by the shRNA-containing lentiviruses. Transduction of two different *SIGLEC1*-specific shRNA (#4 and #5), but not of a non-target shRNA control, led to a drastic decrease in Siglec-1 surface expression in mDC LPS without perturbing the correct differentiation to mDC ($p \leq 0.0001$; Fig. 50 A and B). The downregulation of Siglec-1 expression in mDC LPS also resulted in a concurrent loss of $VLP_{HIV\text{-}Gag\text{-}eGFP}$ capture capacity ($p \leq 0.0008$; Fig. 50 A and B). Furthermore, transduction of a *SIGLEC1*-specific shRNA, but not of non-target shRNA, decreased mDC LPS capacity for HIV-1 *trans*-infection to the $CD4^+$ reporter cell line TZM-bl (Fig. 50 C). This approach of *SIGLEC1* knockdown complement the results obtained with the inhibition by α-Siglec-1 mAb 7D2, strongly supporting the conclusion that Siglec-1 is a central molecule mediating HIV-1 capture and *trans*-infection by DC.

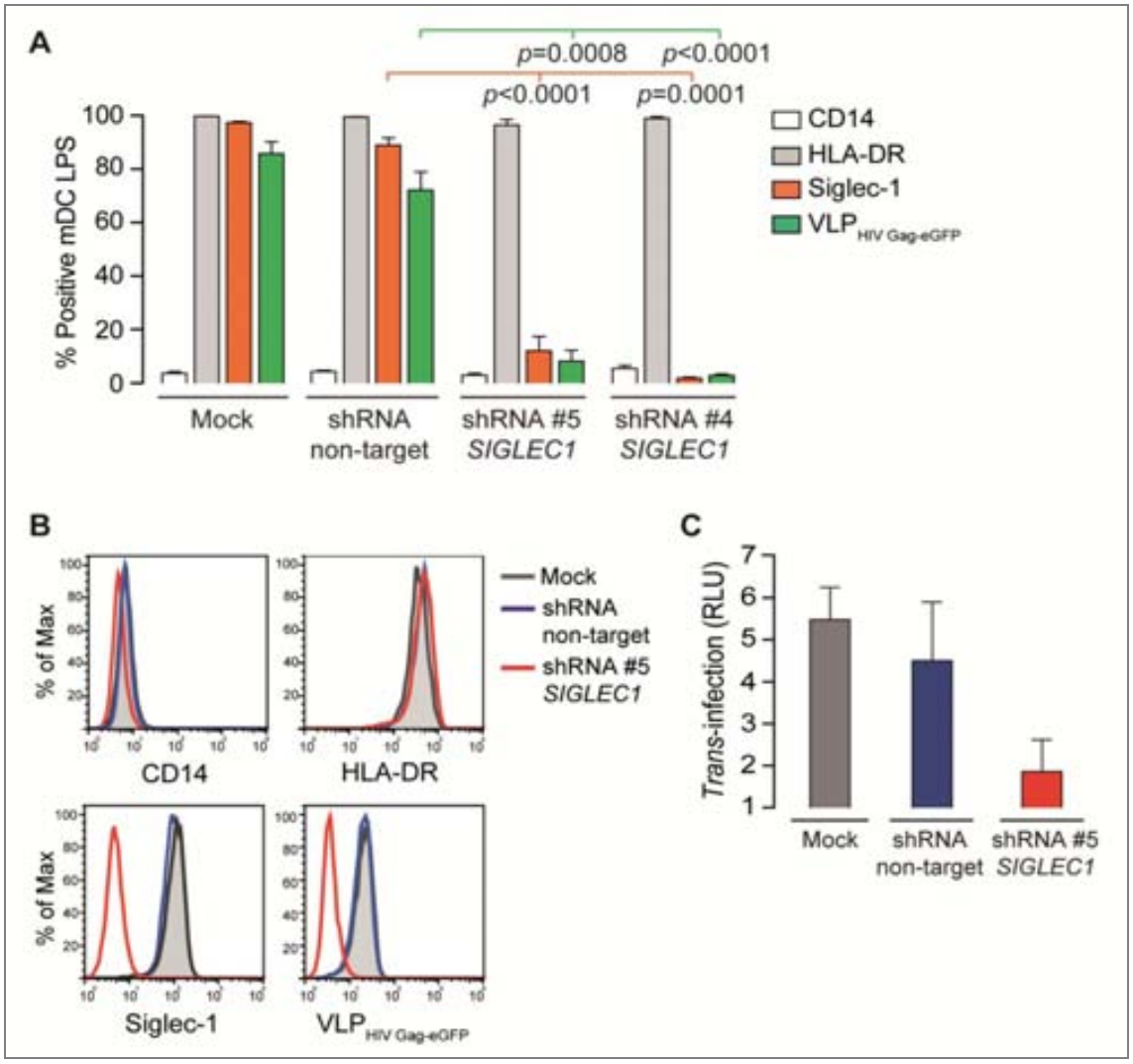


**Figure 50. *SIGLEC1* silencing blocks viral capture and *trans*-infection. (A)** Interference of *SIGLEC1*. Percentage of mDC LPS positive for CD14, HLA-DR, Siglec-1, or $VLP_{HIV\text{-}Gag\text{-}eGFP}$ capture following mock transduction or transduction with non-target shRNA or two different *SIGLEC1*-specific shRNA clones. Data show mean values and SEM from four experiments including cells from at least four donors. Statistical analysis: paired t test. **(B)** Representative cell surface expression levels of CD14, HLA-DR, or Siglec-1 and $VLP_{HIV\text{-}Gag\text{-}eGFP}$ capture profile of mDC LPS transduced with non-target shRNA (blue), *SIGLEC1*-specific shRNA (red), or mock-transduced (grey). **(C)** HIV-1 transmission to $CD4^+$ reporter cell line TZM-bl of mDC LPS that had been mock-transduced or transduced with non-target or *SIGLEC1*-specific shRNA. DC were pulsed with $HIV_{NL4\text{-}3}$ for 5 hours at 37ºC in 5% $CO_2$, washed, and co-cultured with reporter cells for 48 hours. HIV-1 infection of TZM-bl cells was determined by induced luciferase activity in RLU. Data show mean values and SEM from two experiments including cells from four donors.

# Chapter 8

# DISCUSSION

## 1. General overview

This thesis has addressed different questions regarding the interactions of HIV-1 with DC, focusing on HIV-1 capture, antigen presentation and viral transmission. First, we examined the ability of different DC subsets to process and present HIV-1–derived antigens to HIV-1–specific $CD4^+$ and $CD8^+$ T cells, thus assessing different DC maturation stimuli as well as a non-replicative integrase-deficient viral isolate. We simultaneously evaluated the impact of the time lag between DC maturation and antigen loading on the stimulation of HIV-1–specific T lymphocytes, thinking in an immunotherapeutic approach. Secondly, we investigated the DC–T-cell synapses where HIV-1 transmission takes place, exploring the contribution of HIV-1 Env glycoprotein, adhesion molecules and antigen presentation during the formation of these cellular conjugates. And finally, we identified the molecular determinants involved in HIV-1 Env-independent binding and internalization mediated by mDC LPS.



Throughout the course of the investigation we tried to shed light on the relative contribution of mDC during HIV-1 pathogenesis. Upon stimulation with LPS and some proinflammatory cytokines, hallmarks of the immune activation and bacterial translocation during HIV-1 infection, DC mature and adopt an enhanced capacity to capture HIV-1 particles in an Env-independent manner through recognition of viral sialyllactose-containing gangliosides. Siglec-1 is the DC receptor responsible for this sialyllactose-dependent recognition and targets HIV-1 particles to a specific route to be transmitted to $CD4^+$ T cells, thus significantly limiting viral antigen degradation. Consequently, the great HIV-1 uptake ability of mDC LPS results in a poor stimulation of HIV-1–specific $CD4^+$ and $CD8^+$ T cells, demonstrating dissociation between the capacity to capture HIV-1 and to present viral antigens. Furthermore, HIV-1 virions preserve their infectivity within the DC intracellular compartments and gain access to $CD4^+$ T cells taking advantage of the immunological DC–T-cell contacts. Accordingly, HIV-1 does not modulate the formation of these DC–T-cell contacts, and can hijack both antigen-independent and antigen-dependent contacts, essential for maintenance of T-cell homeostasis and initiation of adaptive immune responses, respectively. In addition, the immune activation driven by these DC–$CD4^+$ T-cell contacts boosts productive DC-mediated HIV-1 *trans*-infection, likely contributing to the pathogenesis of HIV-1 infection.

In the following, each part of the results chapters will be discussed in detail in order to arrive at the conclusions for this work.

## 2. Chapter 4 - RESULTS I

### HIV-1 capture and antigen presentation by dendritic cells: enhanced viral capture does not correlate with better T-cell activation

DC play a pivotal role in the generation and regulation of immune responses, and they might also facilitate *in vivo* viral dissemination during HIV-1 infection. The classic DC paradigm proclaims that iDC have abundant endocytic capacity and antigen processing activity but reduced ability to activate T cells, whereas mDC show low endocytic activity with enhanced antigen presentation and immunostimulatory function [29]. In contrast, mDC LPS have greater ability to uptake and transmit viral particles than do iDC [140, 169, 186]. In fact, it has recently been shown that *M. tuberculosis* is able to mature DC promoting similar phenotype of HIV-1 *trans*-infection and viral sequestration to those seen in LPS-stimulated DC, while suppressing class II antigen processing [247]. Although both agonists would mature DC primarily by MyD88-dependent TLR mediated mechanisms, little is known about the fate of captured particles in mDC LPS and whether they represent a source of viral antigens for HLA loading and T-cell activation. Therefore, we assessed the ability of mDC LPS to present HIV-1–derived antigens to HIV-1–specific $CD4^+$ and $CD8^+$ T cells. In order to apply a more clinically oriented approach, we also analyzed DC matured with the proinflammatory cocktail ITIP, the standard stimulus for DC maturation in immunotherapy. Furthermore, we evaluated a non-infectious integrase-deficient HIV-1 isolate, $HIV_{NL4\text{-}3\Delta IN}$, to maximize antigen loading of DC while blocking DC-mediated *trans*-infection of HIV-1.

We first confirmed that maturation of DC with LPS (mDC LPS) increased subsequent HIV-1 capture and *trans*-infection compared with iDC. Surprisingly, although mDC LPS and mDC ITIP displayed a similar phenotypic profile (regarding DC-SIGN, CD80, CD83, CD86, as well as HLA-A, -B, -C, and -DR), maturation of DC with ITIP did not improve the uptake or transmission of $HIV_{NL4\text{-}3}$. Moreover, both cell types exhibited distinct intracellular localization of viral particles by confocal microscopy. Consistent with our $p24^{Gag}$ ELISA results, mDC LPS displayed a large sac-like compartment where viral particles were concentrated, while mDC ITIP showed a random distribution of HIV-1, similar to that observed in iDC. Several activation signals have been used for DC maturation, including Poly I:C, R848, LPS, IFN-γ, CD40L, and TNF-α; however, although all maturation stimuli confer a mature phenotype, the functional ability of the resulting matured DC for polarization, secretion, or migration of $T_H$ function varies[140, 184]. In addition, comparable results to those of iDC were

obtained when DC were matured with LPS (iDC+LPS) or ITIP (iDC+ITIP) during viral uptake, suggesting that complete LPS activation can provide DC with an exceptional ability to capture HIV-1 particles. In fact, DC matured with LPS for 48 hours (our mDC LPS) exhibit maximal viral capture ability, while DC matured for 6 hours (our iDC+LPS) show a viral uptake similar to that of iDC [197]. Altogether, our results indicate that DC-mediated HIV-1 capture and transmission is not only dependent on the DC maturation state [169], but also on the activation stimulus used for maturation [186], as well as the time lag existing between DC maturation and antigen loading.

DC are able to process exogenous antigens and present them through HLA-I and HLA-II for stimulation of $CD8^+$ and $CD4^+$ T cells, respectively. However, we have observed that, despite higher viral uptake and increased HLA molecule expression levels in mDC LPS, this DC condition induced poor stimulation of HIV-1–specific $CD4^+$ and $CD8^+$ T-cell clones. On the contrary, when DC are matured with LPS during viral capture (iDC+LPS), antigen presentation of HIV-1–derived antigens was efficiently triggered through HLA-I and HLA-II pathways. Although iDC+LPS trapped 5 times fewer virions than mDC LPS, they were more efficient in eliciting HIV-1–specific $CD4^+$ and $CD8^+$ T-cell responses. Conversely, DC maturation with ITIP during viral capture (iDC+ITIP) was only able to efficiently activate HIV-1–specific $CD8^+$ T cells but not $CD4^+$ T cells. Like other authors [295], we observed that DC maturation differentially regulated exogenous HLA-I and HLA-II presentation pathways, although in our case, LPS activation of DC during viral uptake facilitated presentation of HIV-1–derived antigens to $CD4^+$ and $CD8^+$ T cells. Our experiments showed that HIV-1–derived antigen presentation was not directly associated with viral capture but was mainly affected by DC status and the activation stimulus used for DC maturation.

Mature DC efficiently trapped more intact viral particles than iDC, with a completely different localization of internalized HIV-1–derived proteins, indicating different intracellular fates for captured virions [181]. Trapped infectious HIV-1 in mDC LPS are concentrated in non-conventional compartments rich in the tetraspanins CD81, CD82, CD9, and CD63, but not in the lysosomal marker LAMP-1 [163, 169, 172, 183]. This large vesicle, not present in iDC, is suggestive of a multivesicular body; in addition, its mildly acidic pH preserves HIV-1 infectivity [163, 169]. However, HLA-II antigen presentation depends on endosomal/lysosomal maturation and acidification [254], and inhibition of endosomal acidification has been reported to increase HIV-1 infectivity [255]. Some studies even show that intracellular HIV-1 degradation occurs faster in iDC than in mDC LPS [149, 163, 169]. Therefore, the low ability to activate

HIV-1–specific $CD4^+$ T cells observed in mDC is probably the result of the accumulation of virions in a non-degrading neutralized endosome, thus protecting HIV-1 infectivity and hampering proper lysosomal degradation. Consequently, intact virions confined in this slow-degrading vesicle in mDC would be routed to a *trans*-infection pathway rather than to antigen presentation, thus enabling dissemination of HIV-1 infection. On the other hand, antigen presentation to $CD4^+$ T cells is initiated upon LPS activation of DC [296, 297] and is dependent on the presence of TLR4 ligands such as LPS with the cargo within the individual phagosome [296]. Furthermore, maturation of DC with LPS activates the vacuolar proton pump which acidifies the lysosomes and facilitates antigen proteolysis and efficient formation of peptide–HLA-II complexes [298]. Our results are consistent with these observations, since DC maturation with LPS—but not with ITIP—during antigen loading notably increases HIV-1–derived antigen presentation to $CD4^+$ T cells.



Envelope integrity and virion functionality are crucial for exogenous presentation of HIV-1 antigens through the HLA-I pathway, as adequate fusion of viral and cellular membranes via CD4 and co-receptor enables cytosolic cleavage of Gag protein by the proteasome [253, 254]. Nevertheless, maturation of DC is associated with a decline in HIV-1 fusion [147], which, in turn, has a direct impact on the ability of mature DC to support viral replication [144, 147]. Besides, other restriction factors such as SAMHD1 [151] or APOBEC3G [155] in mDC have been reported to limit HIV-1 replication upon viral fusion. Consistent with these findings, our results showed that mDC matured with either LPS (mDC LPS) or ITIP (mDC ITIP) had a limited capacity for *cross*-presenting HIV-1–derived antigens, probably owing to the reduced viral fusion in these cell subsets. Interestingly, although iDC capture lower amounts of virions, HIV-1 would be more able to fuse in immature rather than in mDC, facilitating cytosolic degradation of viral antigens. Therefore, these findings support our observations, given that HLA-I antigen presentation of HIV-1–derived peptides was triggered more efficiently when iDC matured during antigen uptake.

Because of the exceptional ability of DC to generate cellular and humoral immune responses, they have been used as tools for immunotherapy of HIV-1 infection [299]. An anti-HIV-1 therapeutic vaccine should induce a specific and efficient immune response against the virus while regulating chronic activation of the immune system. $CD4^+$ T cells are required for the development of cytotoxic $CD8^+$ T cells, which lyse infected cells by HLA-I–dependent mechanisms. Thus, antigen presentation by HLA-I or HLA-II molecules is mandatory for the development of cognate T-cell response

[300]. However, the qualitative response of a DC-based vaccine will be determined primarily by the adjuvants used, as these are DC activators [249, 250]. The most widely used DC maturation stimulus in immunotherapy is the proinflammatory cocktail ITIP [184, 248]; however, our results show that it only boosts HLA-I antigen presentation when DC mature during viral capture. In contrast, maturation of DC with LPS during antigen loading was the best approach when eliciting both HLA-I and HLA-II antigen-specific responses. LPS is a potent adjuvant *in vivo*, although it is not used in clinical practice because of its high toxicity. Furthermore, it has been reported that levels of circulating LPS increase significantly in chronically HIV-1–infected individuals as a result of microbial translocation, contributing to chronic immune activation [187]. However, our results indicate that other TLR-4 ligands are worthy of evaluation. Monophosphoryl lipid A (MPL), a derivative form of lipid A, retains the immunostimulatory activity of LPS but with lower toxicity [301]. MPL was recently successful in mice as an adjuvant in immunization with liposomes containing HIV-1 $p24^{Gag}$, as it induced HIV-1 $p24^{Gag}$–specific $CD8^+$ T cells, effector $CD4^+$ T cells, and cytokines with a $T_H1$–type profile [302]. Moreover, stimulation of human DC with MPL induces maturation, migration, survival signals, and secretion of cytokines [303, 304]. Although MPL-matured DC secrete lower levels of IL-12 than mDC LPS [304], IL-12 secretion can be rescued by maturing DC with MPL and IFN-γ to induce more potent $T_H1$ polarization, which is essential for the induction and maintenance of the $CD8^+$ T-cell response [305]. Although further investigation is required, our iDC+LPS approach seems feasible in an immunotherapeutic context when MPL is used as a maturation stimulus.

Several protocols have been used in DC-based immunotherapy to stimulate the presentation of peptide-HLA complexes with high efficiency. Chemical inactivation with AT-2, which preserves the native morphology of viral particles [306], has provided encouraging immune results in eliciting HLA-I responses [104, 307, 308], but its use in human clinical trials is not approved by European regulatory authorities. Thus, novel delivery tools for cell therapy vaccination and new methods to enhance the immunogenicity and antiviral efficacy of DC-based vaccines for HIV-1 infection are needed. We evaluated $HIV_{NL4\text{-}3\Delta IN}$, an integrase-deficient $HIV_{NL4\text{-}3}$ isolate that lacks the whole integrase coding region. We showed that $HIV_{NL4\text{-}3\Delta IN}$ preserves intact virion morphology and envelope functionality despite not being infectious. Interestingly, in the context of antigen presentation assays, $HIV_{NL4\text{-}3\Delta IN}$ behaves as a wild-type virus, as it is efficiently captured and presented by DC in the absence of viral replication.

Consequently, $HIV_{NL4-3\Delta IN}$ could be an attractive immunogen for future vaccine candidates.

In summary, our results clarify the different intracellular trafficking routes of HIV-1 in DC, diverting the processing and presentation pathway from *trans*-infection depending on DC maturation status and the activation signal used for DC maturation. Moreover, we observed that higher viral capture in DC does not guarantee better antigen presentation or T-cell activation. These results provide new insights into DC biology and have implications in the optimization of DC-based immunotherapy against HIV-1 infection.

## 3. Chapter 5 - RESULTS II

**The infectious synapse formed between mature dendritic cells and CD4$^+$ T cells is independent of the presence of the HIV-1 envelope glycoprotein**

Immune cells communicate with each other through cell-to-cell contact. Viruses such as HIV-1 can take advantage of this contact to amplify viral infection. By hijacking the existing pathways of cell-to-cell communication, HIV-1 can evade certain stages of the humoral immune response [309] and reach the final target of infection, namely, CD4$^+$ T lymphocytes. It has been predicted that the vast majority of HIV-1–infected cells in lymphoid tissue are infected through cell-to-cell transmission [180], since cell-mediated HIV-1 infection is much more efficient than infection by cell-free virus [179]. DC, which are professional APC, are constantly scavenging for pathogens in peripheral tissue and interacting with other immune cells. In addition, mDC provide a perfect microenvironment for potentiating viral dissemination, because they can *trans*-infect HIV-1 by retaining and transmitting infectious virions without becoming infected [76, 145, 169, 177, 183, 186, 273]. In our study, we characterized the molecular interactions at the infectious synapse between mDC harboring HIV-1 and non-activated primary CD4$^+$ T cells where *trans*-infection takes place. We evaluated the contribution of HIV-1, adhesion molecules, and antigen recognition in conjugate formation, viral transmission, and cellular activation and proliferation.

We showed that, unlike virological synapses between productively HIV-1–infected cells and uninfected target cells [191, 192, 198], infectious synapses between DC harboring HIV-1 and uninfected CD4$^+$ T cells did not rely on Env-CD4 interactions. Therefore, the measurement of cellular contacts by flow cytometry showed that uninfected CD4$^+$ T cells established the same percentage of conjugates with mDC, independently of the presence or absence of HIV-1. Although the virological synapse between HIV-1–infected and uninfected T cells could be modulated by adhesion molecules [198], in the infectious synapse, adhesion molecules are important for the establishment of cellular contacts between mDC and CD4$^+$ T lymphocytes. We showed that specific mAb against ICAM-1 and LFA-1 blocked mDC–CD4$^+$ T-cell conjugation. This reduction in cell-to-cell adhesion also resulted in a marked decrease in the productive mDC-mediated HIV-1 *trans*-infection of primary CD4$^+$ T cells. Although ICAM-3 contributes to the initial scanning of T lymphocytes and APC before antigen-specific recognition [310], blockade of ICAM-3 did not impact conjugate formation. Furthermore, specific mAb against ICAM-3 did not affect the transmission of HIV-1

between mDC and $CD4^+$ T cells. This observation is consistent with the findings of other authors, who have demonstrated the relevance of ICAM-1 and LFA-1 [186, 200, 201], but not of ICAM-3 [201], in the DC-mediated transmission of HIV-1 across the infectious synapse. As with the virological synapse [176, 311], and according to the results of other authors [312], we confirmed that the formation of the infectious synapse was an actin-dependent process. Remodeling of the actin cytoskeleton not only enables recruitment of receptors to the interface between mDC and $CD4^+$ T cells to facilitate transmission of HIV-1 [177], but it also enables HIV-1 polarization and sac-like compartment formation in mDC upon viral capture [183, 197].

Mature immunological synapses between mDC and T lymphocytes form as a result of robust cognate pMHC–TcR interaction, co-stimulatory receptors, and adhesion molecules, all of which leads to T-cell activation. Nevertheless, DC and T cells can also establish antigen-independent contacts [122], which, in the same way as the immunological synapses, are initiated by means of adhesion molecules [120, 121]. Engagement between ICAM-1 and LFA-1 facilitates the pMHC-TcR interactions in immunological synapses [265], providing and consolidating positional stability to enhance T-cell sensitivity to antigen [313]. In the absence of antigen-specific recognition, these interactions result in the recruitment of molecules involved in the immunological synapse (eg, the HIV-1 receptors CD4, CXCR4, and CCR5 on the T cell) to the contact zone and in low levels of signaling in the T cell [122, 177]. Moreover, in the presence of HIV-1, these contacts lead to polarization of the HIV-1–containing compartment in DC, thus facilitating viral transmission through the infectious synapse [149, 177]. We confirmed that ICAM-1 and LFA-1 play a significant role in the infectious synapse, given that the blockade of these adhesion molecules equally affected autologous and allogeneic mDC–$CD4^+$ T-cell co-cultures. However, the MLR in allogeneic co-cultures or the presence of SEA did not increase the percentage of cellular contacts compared to autologous co-cultures, indicating that MHC-TcR recognition did not increase the number of mDC–$CD4^+$ T-cell conjugates. In contrast to the minor role in cell conjugation, the sustained MHC-TcR binding in allogeneic co-cultures and the presence of SEA boosted the productive DC-mediated HIV-1 *trans*-infection of $CD4^+$ T cells through the infectious synapse by a mechanism that is strongly associated with the immune activation mediated by mDC through TcR signaling and co-stimulation.

Although the decisive factor for efficient HIV-1 transmission is co-receptor expression in the different T-cell subsets [314], the presence of DC enhances

susceptibility to HIV-1 infection and replication in target cells [169, 183, 186, 201, 314]. The activation status and proliferation of T cells can have a considerable effect on the infectivity and replication of HIV-1. Antigen recognition, such as in alloantigen- or nominal antigen–specific interactions between DC and $CD4^+$ T lymphocytes, induces full T-cell activation and proliferation through TcR and co-stimulatory signaling [122, 266]. Nevertheless, contact between DC and $CD4^+$ T cells in the absence of cognate antigen can also lead to a series of T-cell responses, such as weak proliferation and long-term survival, which are crucial for maintenance of homeostasis in the naïve T-cell pool *in vivo* [122]. In our experiments, mDC were able to *trans*-infect HIV-1 to autologous non-activated primary $CD4^+$ T cells in the absence of nominal antigen, thus inducing lower levels of T-cell activation and proliferation. Consequently, HIV-1 could take advantage of labile mDC–$CD4^+$ T-cell contacts, without antigen-specific recognition, to infect $CD4^+$ T cells and potentially contribute to viral dissemination and HIV-1 latency. On the other hand, we have shown that allogeneic co-culture of mDC and $CD4^+$ T lymphocytes induced higher levels of CD69 and CD25 expression and proliferation, independently of the presence of HIV-1, and that it resulted in higher viral *trans*-infection and replication in $CD4^+$ T cells. Thus, stable cellular conjugates of DC and $CD4^+$ T cells, such as those found in alloantigen or antigen-specific contacts, would enhance viral transmission across the synapse by increasing the susceptibility of target cells.



In summary (Fig. 51), the initial stages of contact between mDC and $CD4^+$ T cells are dependent on the adhesion molecules ICAM-1 and LFA-1. These interactions facilitate the recruitment of MHC, TcR, CD4, CXCR4, CCR5, and other molecules to the contact zone. Then, if cognate pMHC-TcR recognition occurs, cellular interactions consolidate into a mature immunological synapse, providing vigorous TcR and co-stimulatory signaling. When mDC harbor HIV-1, the virus could exploit these pre-existing cellular contacts to infect $CD4^+$ T cells without perturbing the formation of cell conjugates. Consequently, either the contact between mDC and $CD4^+$ T cells or the mature immunological synapse become infectious synapses. Furthermore, antigen-specific recognition would increase T-cell activation and, as a result, the susceptibility of $CD4^+$ T cells to productive HIV-1 infection. Therefore, immunological synapses turn into potent infectious synapses, thus explaining why HIV-1 preferentially infects antigen-specific $CD4^+$ T cells [274-276].

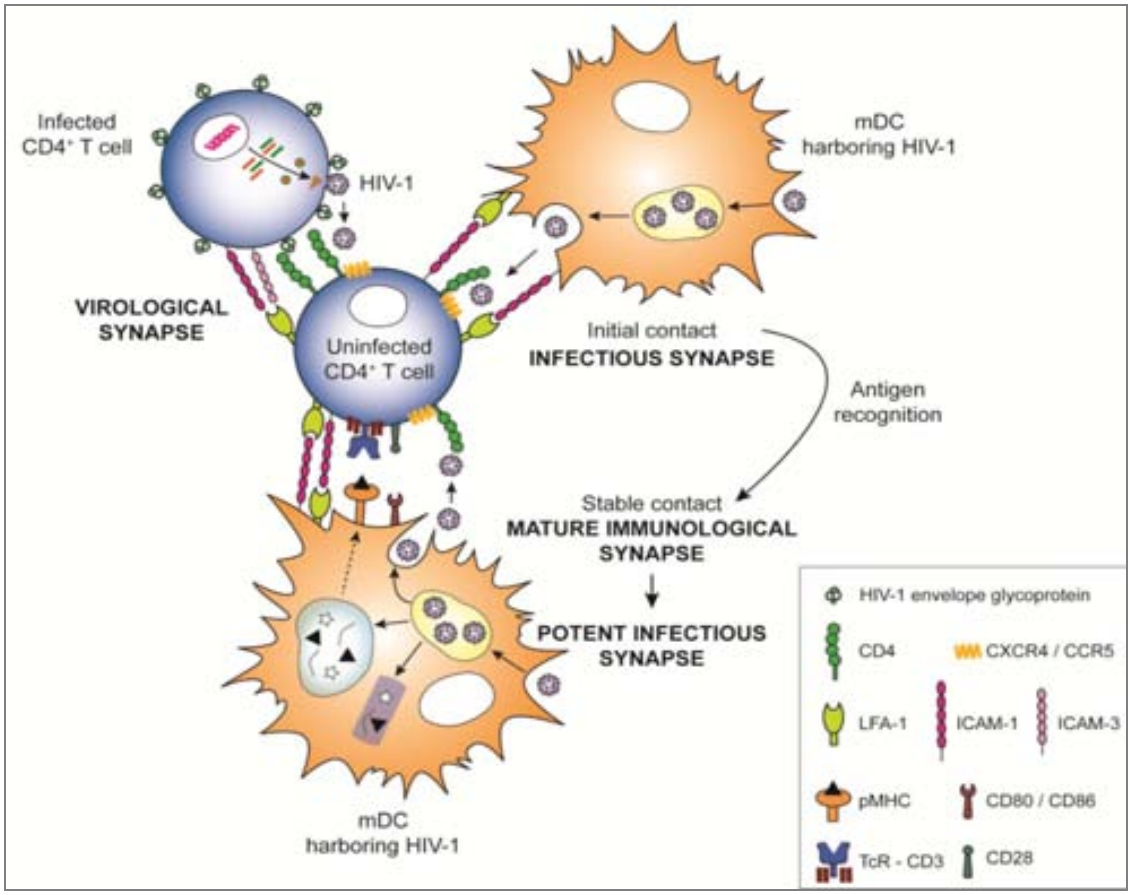


**Figure 51. Comparison between the formation of virological, infectious, and immunological synapses.** Different molecules are involved in the formation of these synapses, although they all enable infection of $CD4^+$ T lymphocytes by HIV-1. The virological synapse relies on engagement between the Env on the surface of an HIV-1–infected cell with the CD4 molecule in the uninfected target cell and on adhesion molecules such as ICAM-1, LFA-1 and ICAM-3. On the other hand, the initial contact between an mDC and a $CD4^+$ T cell is dependent on the adhesion molecules ICAM-1 and LFA-1. If cognate pMHC-TcR recognition occurs, contact between mDC and T cells stabilizes, thus constituting a mature immunological synapse. This antigen-specific signaling also involves co-stimulatory receptors and adhesion molecules and induces strong T-cell activation. When mDC harbor HIV-1, contacts between mDC and T cells would become infectious synapses, since HIV-1 could exploit the pre-existing cell-to-cell interactions to gain access to the target cells without modulating conjugate formation. Therefore, in the context of antigen-specific recognition between mDC harboring HIV-1 and T lymphocytes, immunological synapses would turn into potent infectious synapses by increasing the susceptibility of $CD4^+$ T cells to productive HIV-1 infection. For simplicity, additional DC–T-cell interactions are not depicted.

## 4. Chapter 6 - RESULTS III

**Sialyllactose in viral membrane gangliosides is a novel molecular recognition pattern for mDC LPS capture of HIV-1**

Maturation of DC with certain proimflammatory mediators, such as LPS, increases their ability to capture HIV-1 in a gp120-independent manner [144, 169, 172, 173]. This mechanism was thought to rely on GSL content of HIV-1, since viral uptake by mDC LPS was abrogated when HIV-1 particles were produced in GSL-deficient cells [171, 172]. Nevertheless, the molecular determinants of HIV-1 responsible for this Env-independent interaction were still uncharacterized. In this study we have identified the sialylated gangliosides in the membrane of HIV-1 as decisive factors for specific capture by mDC LPS. Therefore, this gp120-independent viral capture is dependent on exposed sialyllactose moiety on gangliosides, which acts as molecular recognition pattern. The ganglioside GM3 was previously detected in the membrane of HIV-1 and several other viruses, such as simian foamy virus (SFV), vesicular stomatitis virus (VSV), or murine leukemia virus (MLV) [286, 287]. In addition, we have also characterized for the first time the presence of GM1, GM2, and GD1 on the HIV-1 membrane. Gangliosides are significant components of the plasma membrane lipidome [287], suggesting that all enveloped viruses, which bud from the plasma membrane of infected cells, may be captured into mDC by the reported mechanism unless they exclude sialyllactose-containing gangliosides.

Given that retroviruses acquire their lipid envelope during budding from the plasma membrane of the producer cell, viral ganglioside content may vary depending on the membrane composition of the host cell. $CD4^+$ T cells and macrophages, which are the primary target cells for productive HIV-1 infection, display high levels of GM3 [286, 315], and this expression can be modulated by cell cycle and cell activation status [316]. Thereby, the GM3 biosynthesis is dramatically upregulated upon differentiation of macrophages [317] or activation of $CD4^+$ T cells with proinflammatory mediators [318]. Intriguingly, HIV-1 infection can also impact on the cellular GSL content by increasing the cell surface expression of GM3 on PBMC [319]. Remarkably, HIV-1–infected patients show enhanced GM3 content in the plasma membrane of T lymphocytes and high titers of anti-lymphocytic GM3 antibodies [320, 321]. Consequently, the immune activation characteristic of HIV-1 infection may contribute, on the one side, to increase the ganglioside composition of HIV-1 producer cell and, on the other side, to enhance the ability of DC to capture and *trans*-infect HIV-1 [169, 173, 186] in a gp120-independent sialyllactose-dependent manner. Thus viral replication

may lead to the insertion of an increased amount of distinct gangliosides into virions, affecting mDC recognition and local immunosurveillance.

The potential immunological role of mDC uptake implies efficient antigen capture and processing throughout the antigen presentation pathway. Interestingly, antigen-bearing cellular secreted vesicles known as exosomes also follow the same trafficking route as HIV-1 in mDC LPS [172] and contain gangliosides such as GM3 or GM1 [322]. Hence, sialyllactose-carrying gangliosides in the membrane of viruses and cellular vesicles are targeting molecules for mDC uptake, a pathway that may normally lead to antigen presentation and has been subverted by HIV-1 for infectious virus storage and transmission. Exosomes act as antigen messengers between immune cells, amplifying the initiation of adaptive immune responses [323]. They can serve as source of antigen, but can also mediate direct antigen presentation to T lymphocytes when mDC are present [323]. Thus, HIV-1 hides from the immune system by mimicking the exosome ganglioside composition, gaining access to the exosome *trans*-dissemination pathway intrinsic to mDC [172]. After recognition of sialyllactose-carrying gangliosides in HIV-1 by mDC, the virus goes to a tetraspanin-rich compartment of the DC away from lysosomal degradation [163, 169, 172, 181] that finally flows to the DC–T-cell synapse [172, 177]. Consequently, although myeloid cells are largely refractory to productive HIV-1 infection due to the presence of cellular restriction factors such as the recently identified SAMHD1 [150, 151], this sialyllactose driven HIV-1 *trans*-infection process seems to exploit a pre-existing cellular trafficking machinery that avoids the activation of these intrinsic immune pathways.

The efficient capture of ganglioside-carrying cellular vesicles or virions suggests a model where a specific receptor present on the cell surface of mDC (and possibly other cells) recognizes the sialyllactose moiety on virions or vesicle membranes. Gangliosides have been reported to function as cell adhesion molecules [324], and as host cell receptor for several viruses [325-327] and for human toxins [328]. Specific recognition of vesicular gangliosides would then trigger uptake of the respective particles into an intracellular compartment from where they may be either recycled to the surface, as in HIV-1 transmission to $CD4^+$ T cells, or fed into the antigen presentation pathway. Disappointingly, mDC LPS, which exhibit great HIV-1 uptake through sialyllactose interaction, display limited capacity for presenting HIV-1–derived antigens [*see* Chapter 4 - RESULTS I *and* [273]], indicating that HIV-1 exploits DC for viral persistence and immune evasion.

The results of this study identify sialyllactose on membrane gangliosides as a relevant molecular recognition pattern for mDC, explaining the specificity of this process and providing the basis for its future exploitation for interventional or vaccine purposes. These findings have also been corroborated by other study groups [291].

## 5. Chapter 7 - RESULTS IV

**Siglec-1 is a novel DC receptor that mediates HIV-1 capture and *trans*-infection through recognition of viral membrane gangliosides**

DC-SIGN was initially proposed as the HIV-1 attachment factor concentrating virus particles on the surface of DC [76], targeting the virus to intracellular compartments evading lysosomal degration and allowing for viral persistence [329]. However, later studies showed a variable contribution of DC-SIGN to HIV-1 capture and *trans*-infection [204], since it was demonstrated that HIV-1–targeting by DC-SIGN leads to degradative pathways following MHC-I and MHC-II presentation of HIV-1–derived antigens [104, 165, 166]. Furthermore, maturation of DC with activation stimuli such as LPS or IFN-α downregulates DC-SIGN expression but concurrently enhances their efficiency in capturing and *trans*-infecting HIV-1 independently of the viral Env glycoprotein [169-172].



After identification of sialyllactose as the determinant in HIV-1 membrane responsible for this Env-independent attachment to mDC LPS [*see* Chapter 6 - Results III and [290]], we sought to determine the DC receptor to which sialyllactose-containing gangliosides bind to. Three lines of evidence identified Siglec-1 as a novel DC receptor for HIV-1 capture and *trans*-infection. First, Siglec-1 expression correlated with viral capture and *trans*-infection capacity of DC. Second, mAb against Siglec-1 specifically inhibited HIV-1 capture in a dose-dependent manner. And finally, *SIGLEC1* knockdown reduced viral capture and *trans*-infection, while heterologous de novo expression of Siglec-1 enhanced HIV-1 capture and *trans*-infection. Interestingly, a potential contribution of Siglec-1 in HIV-1 infection has been previously reported by other studies showing an increased surface expression of this molecule on $CD14^+$ monocytes and macrophages during HIV-1 infection [330-332]. However, these studies analyzed Siglec-1 interactions with sialylated viral envelope proteins, while our results clearly show that HIV-1 capture depends on sialyllactose on viral membrane gangliosides interacting with Siglec-1, but does not require viral glycoproteins.

Our results indicate that both DC-SIGN and Siglec-1 contribute to *trans*-infection by iDC, while HIV-1 capture by highly *trans*-infecting mDC LPS is independent of DC-SIGN and mainly requires Siglec-1 (Fig. 52). Hence, although Siglec-1 viral binding via sialyllactose recognition does not discriminate between infectious or non-infectious HIV-1 particles, the greater the expression of Siglec-1, the greater the amount of virions captured and transmitted by DC, diminishing the relative contribution of DC-

SIGN gp120-mediated viral capture to *trans*-infection. Given that lectins such as DC-SIGN and Siglec-1 generally achieve high avidity binding by clustering of both receptor and ligand [292], recognition of thousands of sialyllactose containing gangliosides in the viral membrane by Siglec-1 should be clearly superior to the interaction of DC-SIGN with only 14±7 timeric gp120 spikes per virion [294]. Siglec-1 is the only Siglec family member tested that mediated HIV-1 capture, although all Siglecs interact with sialic acid through their respective V-set domains. This could be caused by different specificities, but is most likely due to Siglec-1 containing the largest number of Ig-like C2-type domains of all Siglecs; these domains act as spacers separating the ligand-binding site from the cell surface. Therefore, Siglec-1 extends beyond the glycocalix of the cell, and is thus available for interaction with external ligands, while other family members mainly bind ligands in *cis* [292].

Although Siglec-1 expression is restricted to myeloid cells, particularly to tissue macrophages found in secondary lymphoid tissues [333, 334], its expression can be rapidly induced and upregulated once myeloid cells are activated [335]. Indeed, DC exhibit a characteristic mature phenotype in HIV-1 viremic patients [336], and upregulation of Siglec-1 on mDC is therefore likely to play an important role in HIV-1 dissemination in lymphoid tissues, thus contributing to HIV-1 disease progression. DC maturation is more likely a consequence of factors released upon HIV-1 infection, which also impact on the composition of plasma membrane of HIV-1 producer cells modulating the GSL content of viral membrane [317-319]. It has been shown that plasma LPS levels are significantly augmented in chronically HIV-1–infected patients due to the increased microbial translocation from the gastrointestinal lumen [187]. In addition, opportunistic co-infections with gram-negative bacteria during HIV-1 infection may also contribute to increase circulating LPS levels [188, 189]. Thus, in this proinflammatory milieu associated with HIV-1 infection and immune activation, LPS may facilitate HIV-1 progression by local and systemic stimulation of DC, which leads to Siglec-1 upregulation and enhanced viral spread.

This work together with several other recent reports indicates that HIV-1 uses a highly sophisticated strategy to evade DC immune surveillance and facilitate disease progression (Fig. 52). Viral capture through Siglec-1 on the mDC surface is beneficial for viral spread through *trans*-infection, but could also be detrimental for the virus if leading to successful antigen presentation. However, captured HIV-1 do not appear to reach the endolysosomal compartment of mDC LPS [163], where antigen processing occurs. Consequently, the enhanced HIV-1 capture by mDC LPS results in higher viral

transmission to target cells but poor antigen presentation to of HIV-1–specific $CD4^+$ and $CD8^+$ T cells [*see* Chapter 4 - Results I, and reference [273]]. Furthermore, interaction of HIV-1 with DC-SIGN can cause downregulation of MHC class II molecules and interferon genes, impairing antiviral immune responses while triggering infectious synapse formation [337]. If productive fusion of the viral and cellular membrane occurs, HIV-1 replication is blocked by the myeloid-specific restriction factor SAMHD1 [150, 151], thus preventing viral antigen production. On the other hand, if DC resistance to infection is bypassed, the interaction of newly synthesized HIV-1 proteins with a cell-intrinsic sensor elicits antiviral immune responses, not typically engaged owing to the absence of DC infection [338].



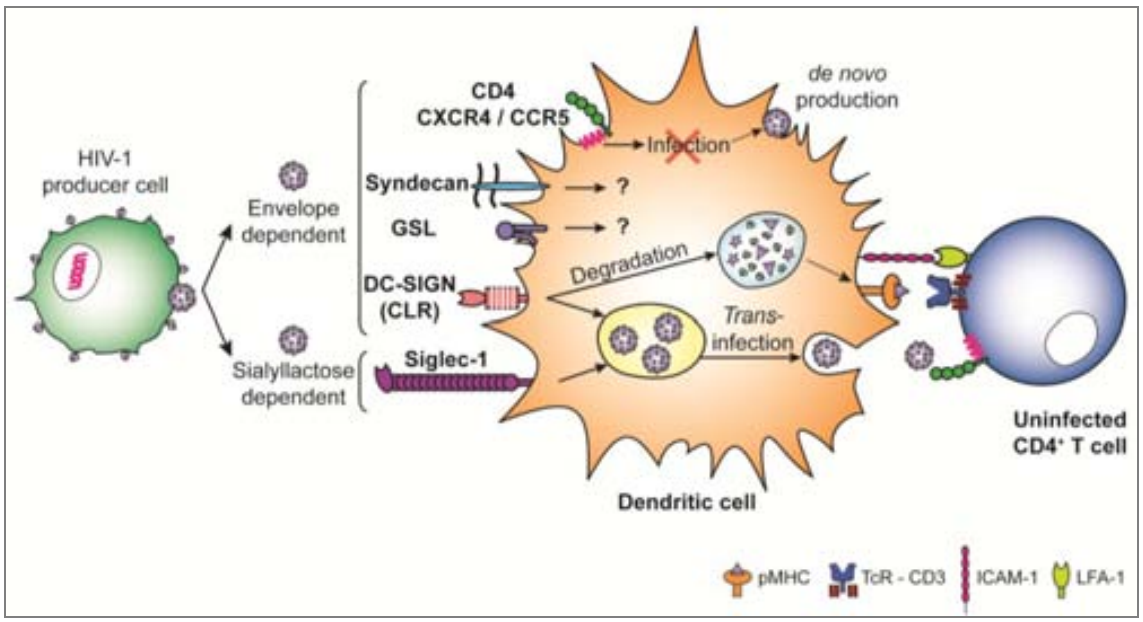


**Figure 52. Model of HIV-1 interaction with DC.** Siglec-1 recognition of HIV-1 by a gp120-independent sialyllactose-dependent mechanism traffics the virus particles to a tetraspanin-rich compartment within DC. Upon DC–T cell contact, this HIV-1 containing vesicle is polarized to the infectious synapse, leading to final *trans*-infection of $CD4^+$ T cells. DC-driven immune stimulation together with the immune activation hallmark of HIV-1 infection increase the susceptibility of targets cells to productive infection. Exposure of producer cell to proinflammatory mediators enhances cell membrane expression of gangliosides, resulting in an increased incorporation of sialyllactose-containing gangliosides in HIV-1 particles. In addition, DC maturation with LPS or certain antiviral cytokines, such as IFN-α, improves their efficiency for capturing and *trans*-infecting HIV-1 by upregulating Siglec-1 expression. *Adapted from Puryear, W.B. and Gummuluru, S., 2013* [161].

Siglec-1 captures HIV-1 through its interaction with sialyllactose-containing gangliosides exposed on viral membranes, and therefore functions as a general recognition receptor for vesicles carrying sialyllactose in their membrane. These include exosomes [322] and probably other sialyllactose-containing viruses. Since

Siglec-1 has been shown to efficiently capture VSV *in vivo*, facilitate antiviral responses and prevent viral neuroinvasion [339, 340], Siglec-1-dependent viral capture may be important for enhancement of immune recognition by direct infection of DC, thus benefiting the host. The observation that Siglec-1 also captures exosomes suggests that this pathway, which normally leads to antigen presentation and intercellular communication, [323] has been hijacked by HIV-1 for infectious virus storage and spread. The discovery of the role of Siglec-1 in capturing sialylated viruses expands our understanding of HIV-1 transmission mechanisms and warrants novel therapeutic approaches aimed to prevent viral dissemination.

# Chapter 9

# CONCLUSIONS

**Objective 1:** To assess the efficiency of mDC LPS for presenting HIV-1–derived antigens to HIV-1–specific $CD4^+$ and $CD8^+$ T-cell clones, comparing with another maturation stimulus and evaluating the impact of the time lag between DC maturation and antigen loading on the stimulation of HIV-1–specific T-cell clones.

**1.1.** Greater HIV-1 uptake by mDC LPS results in poor HIV-1–derived antigens to HIV-1–specific $CD4^+$ and $CD8^+$ T-cell clones and in enhanced viral *trans*-infection to target cells. Consequently, higher HIV-1 capture of DC does not guarantee better antigen presentation or T-cell activation, demonstrating dissociation between the capacity to capture HIV-1 and to present viral antigens.

**1.2.** Although HIV-1 capture is much lower than in fully matured mDC LPS, maturation of DC with LPS during, but not before, viral uptake enhances HIV-1–derived antigen presentation to both HIV-1–specific $CD4^+$ and $CD8^+$ T-cell clones. Therefore, DC maturation state and time lag between DC maturation and antigen loading impact HIV-1 capture and HIV-1–derived antigen presentation.



**Objective 2:** To molecularly characterize the integrase-deficient HIV-1 isolate $HIV_{NL4\text{-}3\Delta IN}$ as an immunogen, also analyzing the efficiency of DC for capturing and presenting $HIV_{NL4\text{-}3\Delta IN}$-derived antigens to HIV-1–specific $CD4^+$ and $CD8^+$ T-cell clones.

**2.1.** Despite lacking the whole integrase-coding region, the $HIV_{NL4\text{-}3\Delta IN}$ displays a comparable morphology to that observed in a wild-type HIV-1. The $HIV_{NL4\text{-}3\Delta IN}$ preserves its envelope integrity and functionality, being as fusogenic as $HIV_{NL4\text{-}3}$, but in the absence of viral replication.

**2.2.** The $HIV_{NL4\text{-}3\Delta IN}$ is captured and presented by DC to HIV-1–specific $CD4^+$ and $CD8^+$ T-cell clones with the same efficiency that a wild-type $HIV_{NL4\text{-}3}$, following the same intracellular trafficking in DC and in the absence of viral *trans*-infection.

**Objective 3:** To explore the contribution of HIV-1 Env in the formation of conjguates between mDC and $CD4^+$ T cells at the infectious synapse and in the subsequent mDC-mediated HIV-1 *trans*-infection, analyzing its role in combination with adhesion molecules and antigen presentation.

**3.1.** Conjugate formation between mDC and $CD4^+$ T cells at the infectious synapse is independent of the presence of the HIV-1 envelope glycoprotein.

**3.2.** Interaction between ICAM-1 to LFA-1 is necessary for both formation of mDC–$CD4^+$ T-cell conjugates and efficient mDC-mediated HIV-1 *trans*-infection.

**3.3.** Antigen recognition or sustained MHC-TcR interaction itself does not mediate cellular conjugation, but boosts productive DC-mediated HIV-1 *trans*-infection of primary $CD4^+$ T lymphocytes by promoting T-cell activation and proliferation.

**Objective 4:** To investigate the molecular determinants involved in the HIV-1 Env-independent binding and internalization mediated by mDC LPS.

**4.1.** The sialyllactose on HIV-1 membrane gangliosides is the molecular determinant required for efficient Env-independent binding, capture and internalization of HIV-1 by mDC LPS.

**Objective 5:** To identify the surface receptor on mDC LPS that enhances their Env-independent uptake of HIV-1 and their capacity to *trans*-infect HIV-1 to $CD4^+$ T cells.

**5.1.** The sialic acid-binding Ig-like lectin 1 (Siglec-1, CD169) is the surface receptor on mDC LPS that recognizes the sialyllactose-containing gangliosides exposed on the HIV-1 membrane, mediating their Env-independent capture of HIV-1 and boosting their capacity to *trans*-infect $CD4^+$ T cells.

# Chapter 10

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# Chapter 12

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