Universitat Autònoma de Barcelona

HIV-1 immune activation induces Siglec-1 expression and enhances viral transmission in myeloid cells

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Badalona 12 de Maig de 2016.

Dra. Dolores Jaraquemada Pérez de Guzmán

A la meva familia i amics que m'aguanten

Myeloid cells are key players in the recognition and response of the host against invading viruses. Paradoxically, upon HIV-1 infection, myeloid cells might also promote viral pathogenesis through *trans*-infection, a mechanism that promotes HIV-1 transmission to target cells via viral capture and storage. The receptor Siglec-1 (CD169) potently enhances HIV-1 *trans*-infection and is regulated by immune activating signals present throughout the course of HIV-1 infection, such as interferon α (IFNα). Here we show that IFNα-activated dendritic cells, monocytes and macrophages have an enhanced ability to capture and *trans*-infect HIV-1 via Siglec-1 recognition of viral membrane gangliosides. Monocytes from untreated HIV-1-infected individuals *trans*-infect HIV-1 via Siglec-1, but this capacity diminishes after effective antiretroviral treatment. Furthermore, Siglec-1 is expressed on myeloid cells residing in lymphoid tissues, where it can mediate viral *trans*-infection. Our results strongly support that Siglec-1 is an important molecule that could accelerate HIV-1 transmission in the crowded cellular environment of lymphatic tissues, where many T-cells can contact myeloid cells.

Since no current antiviral therapy blocks Siglec-1-mediated viral dissemination, we hypothesize that designing a novel generation of therapeutic agents against Siglec-1 could pose an interesting approach to reduce viral spread and limit the settlement of viral reservoirs, bringing us closer to a functional cure of HIV-1. We have followed different approaches to obtain Siglec-1 blocking compounds and tested small or multivalent sialyllactose-like compounds and mAbs against Siglec-1. Optimization of these approaches will aid to identify potent Siglec-1 inhibitors to complement current antiretroviral treatments and enhance future therapeutic strategies.

Les cèl·lules mieloides són actors clau en el reconeixement i resposta de l'hoste contra els virus invasors. Paradoxalment, després de la infecció del VIH-1, les cèl·lules mieloides també poden promoure la patogènesis viral a través de la *trans*-infecció, un mecanisme que promou la transmissió del VIH-1 a les cèl·lules diana a través de la captura i emmagatzematge del virus. El receptor Siglec-1 (CD169) augmenta potentment la *trans*-infecció del VIH-1 i es regulat per senyals immune activadores presents al llarg del curs de la infecció del VIH-1, com és l'interferó α (IFNα). Aquí demostrem que cèl·lules dendrítiques, monòcits i macròfags activats amb IFNα tenen l'habilitat de capturar i *trans*-infectar el VIH-1 via el reconeixement per Siglec-1 de gangliòsids presents en la membrana viral. Monòcits de pacients infectats pel VIH-1 sense tractar *trans*-infecten el virus via Siglec-1, però aquesta capacitat es veu disminuïda després d'un tractament antirretroviral efectiu. A més, Siglec-1 s'expressa en cèl·lules mieloides residents en teixits limfoides, on poden mediar la *trans*-infecció viral. Els nostres resultats suporten fermament que Siglec-1 és una mol·lècula important que pot accelerar la transmissió del VIH-1 en l'entorn multicel·lular dels teixits limfàtics, on les cèl·lules T poden contactar amb les cèl·lules mieloides.

Degut a que actualment no existeix una teràpia antiviral que bloquegi la disseminació del VIH-1 mediada per Siglec-1, nosaltres hipotetitzem que el disseny d'una nova generació d'agents terapèutics dirigits a Siglec-1 poden plantejar un enfocament interessant per a reduir la propagació viral i limitar l'establiment de reservoris virals apropant-nos a una cura funcional del VIH-1. Hem seguit diferents estratègies per a obtenir un compost que bloquegi Siglec-1, hem testat compostos petits o multivalents similars a la sialillactosa i anticossos monoclonals contra Siglec-1. La optimització de aquestes estratègies ens ajudaran a identificar inhibidors potents de Siglec-1 per a complementar els tractaments antirretrovirals actuals i millorar futures estratègies terapèutiques.

ABBREVIATIONS COMMONLY USED

α2-3Gal: α2-3 galactose

Ab: antibody

ABS: antibody binding sites

AIDS: acquired immunodeficiency syndrome

APCs: antigen presenting cells

APC: allophycocyanin (flow cytometry)

APC-Cy7: allophycocyanin-Cyanine7 conjugate (flow cytometry)

ART: antiretroviral treatment

AZT: azidothymidine

BDCA: blood dendritic cells antigen

CCR5: C-C chemokine receptor type 5

CLR: C-type lectin receptor

CTL: Cytolitic CD8+ T lymphocytes

CXCR4: C-X-C chemokine receptor type 4

DAPI: 4, 6-diamidino-2-phenylindole

DC: dendritic cell

DC LPS: dendritic cells LPS activated

DC IFN: dendritic cells IFN activated

DC-SIGN: DC-specific ICAM-3-grabbing non-integrin

DMEM: Dulbecco's Modified Eagle Medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DsRNA: double-stranded deoxyribonucleic acid

eGFP: enhanced Green Fluorescent Protein

ELISA: enzyme-linked immunosorbent assay

Env: viral envelope glycoprotein

ESCRT: endosomal sorting complexes required for transport

FACS: flow activating cytometer sorting

FBS: fetal bovine serum

Fc: constant fraction (antibodies)

FcR: receptor of constant fraction (antibodies)

Fig: Figure

FITC: fluorescein isothiocyanate

FSC: forward scatter channel

GALT: gut-associated lymphoid tissue

GFP: green fluorescent protein

GM-CSF: granulocyte-macrophage colony-stimulating factor

GSL: glycosphingolipid

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: human immunodeficiency virus

HIV-1: human immunodeficiency virus type 1

HIV-2: human immunodeficiency virus type 2

HLA: human leukocyte antigen

Ig: immunoglobulin

ICAM-1: intercellular adhesion molecule 1

ICAM-3: intercellular adhesion molecule 3

iDC: immature dendritic cell

IFNα: interferon alfa

IL-4: interleukin 4

IL-6: interleukin 6

ISGs: interferon-stimulated genes

ITIP: interleukin-1 β, tumor necrosis factor α, interleukin-6 and prostaglandin E2

LC: Langerhan cells

LFA-1: leukocyte function-associated antigen

LPS: lipopolysaccharide

LTR: long terminal repeats

LUV: large unilamellar vesicles

MØ: macrophages

MA: matrix

mAb: monoclonal antibody

M-CSF: macrophage colony stimulating factor

mDC: mature dendritic cell

MDDC: monocyte-derived dendritic cell

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

MLV: murine leukemia virus

Mo: monocytes

MOI: multiplicity of infection

MPS: macrophage phagocyte system

mRNA: messenger Ribonucleic acid

MxA: myxovirus resistance protein A

myDC: myeloid dendritic cell

NC: nucleocapsid

Neu5Ac: n-acetylneuraminic acid

NHP: non-human primates

Ni-Nta: nickel-nitrilotriacetic acid (agarose gel)

NK: natural killer cell

NNRTI: non-nucleoside reverse transcriptase inhibitors

NRTI: nucleoside reverse transcriptase inhibitors

NVP: nevirapine

PAMP: pathogen-associated molecular patterns

PBMC: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PCR: polymerase chain reaction

pDC: plasmacytoid dendritic cell

PE: phycoerythrin

PE-Cy5: phycoerythrin-cyanine 5 conjugate

PerCP: peridinin chlorophyll protein

PerCP-Cy5.5: peridinin chlorophyll protein-cyanine 5.5 conjugate

PGE2: prostaglandin E2

PHA: phytohemagglutin

Poly I:C: polyinoisic:polycytidylic complex

PRR: pattern-recognition receptors

PRRSV: porcine respiratory and reproductive syndrome virus

PS: phosphatidylserine

RLUs: relative light units

RNA: ribonucleic acid

RPMI: Roswell Park Memorial Institute medium

RT: room temperature

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard errors of the means

Siglec-1: sialic acid-binding Ig-like lectin 1

SIV: simian immunodeficiency virus

SFV: semliki forest virus

SP1: spacer peptide 1

SP2: spacer peptide 2

SQV: saquinavir

SSC: side scatter chanel

ssRNA: single stranded ribonucleic acid

TCR: T-cell receptor

TGFβ: transforming growth factor β

Th: T helper cells

TLR: Toll like receptor

TNFα: tumor necrosis factor α

TSL: thymic stromal lymphopoietin

VL: viral load

VLP: viral like particles

VSV: vesicular stomatitis virus

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Chapter 1

INTRODUCTION

The immune system coordinates innate and adaptive immune responses to eliminate infectious agents in an efficient and specific way. Dendritic Cells (DCs), monocytes and macrophages are components of the myeloid cell lineage that initiate and modulate innate and adaptive immune responses, and thus have a critical role in limiting invading pathogens.

In this thesis we will focus on how myeloid cells confront invading pathogens and how those pathogens, such as the Human Immunodeficiency Virus (HIV), have developed strategies to evade these defenses. Our work deciphers the interaction between HIV and myeloid cells, and sheds light into the molecular mechanism that HIV uses to exploit myeloid cells through a myeloid cell receptor called Siglec-1. Siglec-1 allows HIV to dodge the immune response, favoring viral dissemination throughout the body and influencing HIV pathogenesis.

1. The immune system and the immune responses

The immune system is composed of a group of tissues, cells and molecules, which have as their main function to protect the organism against infectious agents such as fungi, bacteria and viruses. To fight back against the wide variety of invading pathogens, the immune system has developed multiple strategies to eliminate infectious agents without altering the individual homeostasis by distinguishing self- from non-self. The global responses coordinated by the immune system against infectious agents are both innate and adaptive immune responses [1, 2].

1.1. Innate immunity

The innate immune response is the first line of host defense against infection and tumor cells and has a profound effect on the establishment of adaptive immunity. The innate immune system evolved several strategies of self/non-self discrimination that are based on the recognition of molecular patterns from infectious non-self, as well as normal and abnormal self. These patterns are deciphered by receptors that either induce or inhibit an immune response, depending on the meaning of these signals. These receptors are called pattern recognition receptors (PRRs) (reviewed by [3]). PRRs

such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), AIM2-like receptors (ALRs), C-type lectin receptors (CLRs) and other DNA sensors, recognize invariant molecular structures called pathogen-associated molecular patterns (PAMPs) (e.g., Lipopolysaccharide -LPS-, peptidoglycan, unmethylated CpG-DNA, bacterial lipoprotein, and yeast mannans) that are shared by numerous pathogens, but are not normally expressed in host tissues [1, 4]. TLRs are type I transmembrane glycoproteins that play a key role in the immune response against microbes. TLRs are expressed in various immune cells including macrophages, monocytes, neutrophils, mast cells, eosinophils, DCs, and T cells. Ten human TLRs have been identified to date and are localized on the cell surface such as the case of TLRs 1, 2, 4, 5, 6, 10 or have an endosomal location as TLRs 3, 7, 8, 9. Important TLRs expressed on myeloid cells are TLR4 that recognizes LPS through association with soluble protein MD-2, and endosomal TLR7/9 that recognize viral single stranded RNA (ssRNA) [3, 5]. On pathogen invasion, PRRs trigger the activation of NF-κB, type I interferon (IFN), or other inflammasome signaling pathways on myeloid cells, such as DCs or macrophages. In turn, this leads to the production of a variety of pro-inflammatory and antiviral cytokines and chemokines, as well as the maturation and migration of antigen-presenting cells (APCs), such as DCs, macrophages and monocytes. The activation of APCs is a prerequisite for the induction of adaptive immunity [6].

1.2. Adaptive immunity

DCs, monocytes, and macrophages are specialized in antigen processing and presentation, which initiate and regulate immune responses. Thus, they are often referred to as APCs [7].

Myeloid cells in an immature state act as sensors that capture encountered pathogens into the peripheral tissues. Captured pathogens are shuttled to lysosomes where proteins are chopped to peptides and loaded onto Major Histocompatibility Complex (MHC) class-II molecules that are retained intracellularly for later presentation [8]. Innate responses create an inflammatory microenvironment that prompts antigen presenting cell activation and migration to secondary lymphoid tissues. This process

culminates with cell-maturation; when MHC class II-peptide complexes are transported to the membrane and co-stimulatory molecules (e.g. CD80 and CD86) are expressed on the surface, preparing APCs for competent T cell priming [8][9]. Then, fully mature APCs present processed antigens that are recognized specifically by $CD4^+$T lymphocytes via T Cell Receptor (TCR) [7].

DCs are also able to present antigens derived from endogenous proteins degraded mainly in the cytosol by the proteasome, presenting them to $CD8^+$ T cells in MHC class I molecules, and triggering cytotoxic responses. However, DCs also have the unique capability of presenting exogenous antigens through MHC class I. This process is called cross-presentation, and is necessary for immunity against tumors and viruses (as reviewed in [10]).

By these means, APCs from the myeloid cell lineage are coordinators of the innate and adaptive immune responses, and thus have a critical role in limiting invading pathogens.

2. The myeloid cell lineage or mononuclear phagocyte system (MPS)

Myeloid and lymphoid progenitor cells are the two major cell lineages of the immune system originating from common hematopoietic stem cells in the bone marrow and giving rise to the myeloid cells (monocytes, macrophages, DCs, neutrophils and others) and lymphoid cells (T cells, B cells, natural killer (NK) cells and others), respectively [11].

In the early 1970s, Ralph van Furth, James Hirsch, and Zanvil Cohn described that the mononuclear phagocyte system (MPS) is constituted by monocytes and macrophages and assumed the premise that all macrophages are derived from monocytes [12]. Later, Ralph Steinman and Zanvil Cohn identified and characterized DCs, which were incorporated into the MPS [13]. Since then, monocytes, macrophages and DCs have been grouped together, and named on the basis of their morphology, function and origin. Advances in flow cytometry technology have enabled the study of different surface markers and the discovery of a wide variety of distinct subsets of DCs, monocytes and macrophages. However, it has also revealed that many of the

proposed unique subset markers are in fact shared between distinct cell types [14]. Furthermore, markers of a particular cell subset are not always consistent between mice and humans. This has led to much confusion and debates regarding which subsets represent distinct cell types and which are simply modified versions of the same cell type [15].

Ontogeny studies have recently demonstrated that the MPS are not derived exclusively from monocytes as originally proposed. Indeed, macrophages also arise during embryonic development and DCs also derive from an adult hematopoietic common DC precursor (CDP) too [16–18]. Based on their cellular origin, MPS is composed of three broad families of cells: (i) embryonic progenitor-derived Macrophages, (ii) CDP-derived DCs, which would be subdivided into plasmacytoid DCs (pDCs) and myeloid DCs (myDCs), and (iii) Monocyte-derived Cells, which would include monocyte-derived DC and monocyte-derived macrophages (**Figure 1**). In addition to this precursor-based classification, functional specialization, cellular activation state, micro-anatomical localization, and surface marker expression should also be taken into account to identify cells in a particular study [14, 19, 20]. In this thesis, we have worked with monocyte-derived cells, including monocytes, monocyte-derived DCs and monocyte-derived macrophages, and tissue isolated APCs. Based on functional specialization, we refer to these cells as monocytes, DCs, macrophages, or tonsillar myeloid cells.

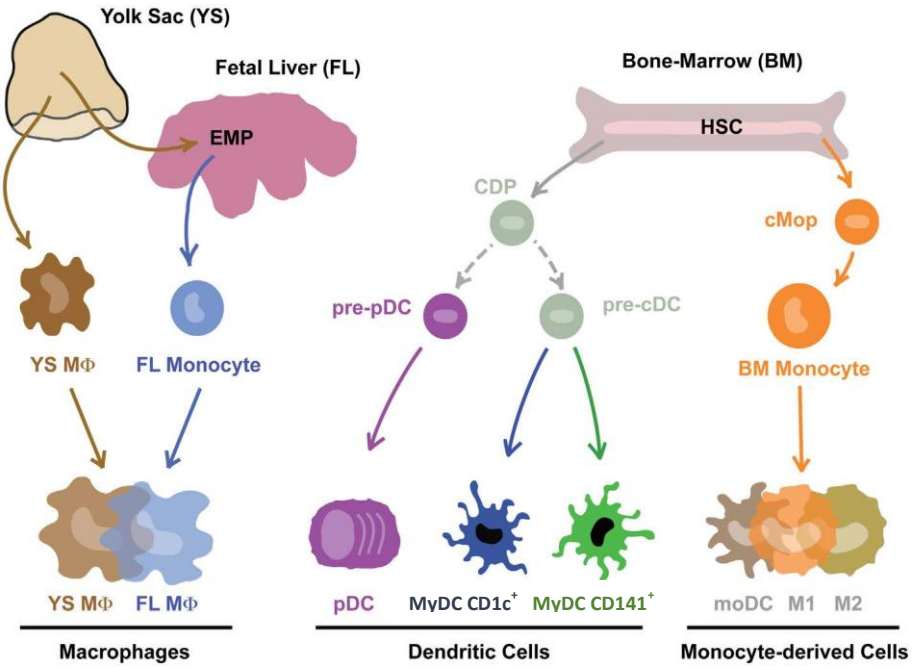


Figure 1. Mononuclear phagocytes and their precursors. EMP: erythro-myeloid progenitors, MØ: macrophage, HSC: haematopoietic stem cells, CDP: common Dendritic cell progenitor, pre-pDC: pre-plasmacytoid dendritic cell, pre-cDC: pre-conventional dendritic cell, pDC: plasmacytoid dendritic cell, myDC: myeloid dendritic cell, cMop: common monocyte progenitor, moDC: monocyte-derived dendritic cell, M1: macrophage type 1, M2: macrophage type 2. Figure from [19].

2.1. Monocytes

Monocytes constitute 2% to 10% of all the leukocytes and are originated from bone marrow precursors (**Figure 1**). They are normally circulating in peripheral blood and their fate depends on the local microenvironment in tissues [20]. Monocytes attach to the endothelium, roll and diapedese into extravascular tissues, where they differentiate into macrophages and DCs, or transiently traverse tissues, maintaining a monocytic profile [21]. In an *in vitro* model of endothelial trafficking without addition of exogenous cytokines, it was observed that monocytes differentiate into DCs, moving from endothelium to the lumen. Those monocytes that remained in the endothelium became macrophages [22, 23]. Monocytes are multifunctional, and have roles in homeostasis, immune defense, and tissue repair (**Table 1**). This cell population is very heterogeneous, has an enormous plasticity, and is able to migrate to tissues.

Three major subsets of blood monocytes have been described in humans, characterized by differential expression of CD14 and CD16 markers. Classical monocytes are described as $CD14^{++}$ $CD16^{-}$ or $CD14^{+}$ $CD16^{-}$, intermediate are defined as $CD14^{++}$ $CD16^{+}$, and non-classical monocytes as $CD14^{+}$ $CD16^{++}$. This latter population is expanded in various infectious diseases, including HIV infection [24].

Monocytes differentiate into DCs upon exposure to granulocyte-macrophage colony-stimulating factor (GM-CSF) plus interleukin-4 (IL-4), or differentiate to macrophages upon exposure to macrophage colony-stimulating factor (M-CSF, also called colony stimulating factor 1 CSF-1; M2 polarization), or upon exposure to GM-CSF (M1 polarization). Therefore, depending on the different cytokines and endothelium milieu, monocytes have three potential fates [20, 25].

2.2. Macrophages

Discovered in 1880 by Elie Metchnikoff, macrophages were the first cellular component of the innate immune system identified. They are originated from the yolk sac or fetal liver precursors, but can also be derived from monocytes (**Figure 1**). Macrophages are a terminally differentiated cell type distributed in almost every tissue of the body: Kupffer cells in the liver, microglia in the central nervous system, metallophilic, and marginal zone macrophages in the spleen, as well as osteoclasts in the bone and alveolar macrophages in the lungs [26]. Macrophages are distinguished as large vacuolar cells that excel in the clearance of apoptotic cells, cellular debris and pathogens. They present pseudopodia, non-specific esterases, and phagocytic granules, which give them a foamy appearance. Their function and phenotype depend on which tissue they reside and the surrounding cytokine milieu. They contribute to several functions during homeostasis, but also during innate and adaptive immune responses. As implied by their name, derived from the Greek *makros* (large) and *phagein* (eat), they are highly efficient phagocytes, and their tasks involve engulfing death cells and clearance of pathogens (**Table 1**) [27].

Owing to the difficulties in isolating primary macrophages in large quantities, in this thesis we have generated macrophages *in vitro* by monocyte differentiation. M-CSF

cultured peripheral blood monocytes remain the predominant *in vitro* system used to generate macrophages [25].

2.3. Dendritic Cells (DCs)

DCs were first identified and characterized in a series of studies examining mouse splenic adherent cells that were published in the 1970s by Ralph Steinman and Zanvil Cohn [13]. They are originated from common DC precursors (CDP) or from bone marrow monocytes (**Figure 1**). DCs, whose name derive from the Greek *Dendron* (tree-like), are defined as cells with a stellate morphology that can efficiently present antigens on MHC molecules and activate naive T cells [28]. DCs orchestrate innate and adaptive immune responses to infection. DCs are a heterogeneous population that can be divided into subsets based on expression of cell-surface markers, anatomical distribution, and immunological function. There are two major subtypes of human DCs: myeloid DCs (myDCs, including myeloid $CD1c^+$ DCs and myeloid $CD141^+$ DCs) and plasmacytoid $CD303^+$ DCs (pDCs) [24, 29, 30].

2.3.1. Plasmacytoid DCs (pDCs)

pDCs are a distinct DC lineage based on morphology, gene expression and ability to secrete high levels of type I IFN following viral encounter (**Table 1**) [31–33]. pDCs are mainly located in blood, lymphoid tissues, and cerebrospinal fluid, but can be recruited to sites of inflammation. In contrast to the “dendritic” appearance of DCs, pDCs exhibit a spherical shape characteristic of antibody-secreting plasma cells. Functionally, pDCs are not phagocytic and have low MHC class II expression, rendering them low ability to present exogenous antigens to $CD4^+$ T cells [34]. They express very high levels of TLR7 and TLR9 that transduce signals from sensing viral nucleic acids that triggers the release of large amounts of type I IFN [29, 35, 36]. pDCs are distinguished from DCs by the lack of CD11c, but the expression of Blood Dendritic Cell Antigen-2 (BDCA-2/CD303/CLEC4C), CD123 (IL3-R) and BDCA-4 (CD304/neuropilin-1) [37]. No *in vitro* model for pDCs is available, and thus they are directly isolated from blood [38].

2.3.2. Myeloid DCs (myDCs)

myDCs are highly phagocytic, specialized antigen-processing and -presenting cells. A key feature of myDCs is their continuous replacement from bone marrow precursors and their ability to secrete high levels of IL-12 (**Table 1**) [7, 39]. DCs express the myeloid cell surface marker CD11c, but lack CD14 or CD16. Blood myeloid DCs can be subdivided depending on differential expression of BDCA cell surface markers as $BDCA1^+$ ($CD1c^+$) or $BDCA3^+$ ($CD141^+$) cells. Unlike pDCs, myeloid DCs express all TLRs except for TLR7 and TLR9, responding well to LPS (TLR4 agonist), flagellin (TLR5 agonist), poly I:C (TLR3 agonist), and R848 (TLR7 agonist) [30].

As regards the anatomical distribution of myDCs in tissues, we distinguish Langerhans cells (LC) and dermal interstitial DCs, which are distributed throughout the mucosal surfaces. LC are located in epidermis and express CD1a and Langerin (CD207), which is absent in dermal interstitial DCs. Dermal interstitial DCs express the mannose receptor (CD206) and DC-SIGN (CD209) [40].

Due to the low frequencies of myDCs *in vivo* (0.5% – 2% of Peripheral Blood Mononuclear Cells, PBMCs), they have been mostly studied using monocyte-derived DCs by culturing monocytes in the presence of GM-CSF and IL-4. Monocyte-derived DCs are a good myeloid DC model, sharing both similar cell surface markers and response to most stimuli. In this thesis, we have used monocyte-derived DCs but will refer to these cells as DCs.

Table 1. Myeloid cell subsets. Adapted from [41, 42].

Cell type	Cellular markers	Primary locations	Average Life Span	Functions/Features
Monocytes	$CD14^{++}$ $CD16^{-}$ $CD14^{+}CD16^{+}$ $CD14^{+}/CD16^{++}$	Peripheral blood	Few days	Patrol blood for surveillance Precursor to macrophages Precursor to DCs Interact with LPS via CD14 Phagocytosis
Macrophages	$CD68^{+}$ $EMR1^{+}$ $CD14^{+}$	Mucosal surface Tissues, Brain (Microglia), Lungs (Alveolar), Liver (Kupffer cells), Bone (Osteoclasts)	Week to months	Phagocytose cellular debris and pathogens Antigen presentation M1/M2 polarization Tissue remodeling
Plasmacytoid Dendritic Cells	$CD11c^{-}$ $CD123^{+}$ $BDCA2^{+}$ $BDCA4^{+}$	Blood, cerebrospinal fluid, and lymph nodes	Days to weeks	Antigen presentation IFNα production upon HIV exposure IDO production
Myeloid Dendritic Cells	$CD11c^{+}$ $CD123^{-}$ $BDCA1^{+}$	Blood, mucosal surfaces, lymph nodes, and cerebrospinal Fluid	Days to weeks	Antigen presentation Phagocytosis DC migration Induce T cells in cell-mediated immunity
Langerhans Cells	$CD1a^{+}$ $Langerin^{+}$	Epidermis, and mucosal Epithelia	Week to months	Phagocytosis Pathogen degradation $CD8^{+}$ T-cell priming and B-cell activation

Despite the presence of an immune surveillance system elegantly coordinated by APCs, only effective antiretroviral treatment can suppress HIV replication, clearly demonstrating that our defenses have failed to control and eliminate HIV infection.

3. Human Immunodeficiency Virus (HIV)

HIV, the etiological agent of the Acquired Immune Deficiency Syndrome (AIDS), is an infectious agent that can cause persistent disease by attacking the immune system, avoiding normal host defense mechanisms, and even subverting these barriers to promote its own replication.

3.1. Epidemiology

HIV has spread worldwide and is now a pandemic infection. According to the World Health Organization, at the end of 2014, 36.9 million of people were living with HIV, of whom 25.8 million were living in Sub-Saharan Africa. That same year, there were 2 million new HIV infections and 1.2 million died of AIDS-related diseases [43].

In 1981, the first clinical observations of AIDS were reported in the United States and then quickly around the world [44]. In 1983, a new human retrovirus, at the time named lymphadenopathy-associated virus (LAV) was isolated from a lymph node biopsy of a patient with generalized lymphadenopathy [45]. Within a year, two distinct groups isolated the novel retrovirus and a serological test was developed to carry out large sero-epidemiological studies, which confirmed that HIV causes AIDS [46–48]. They called it human T-lymphotropic virus type III (HTLV-III) and AIDS-associated retrovirus. To eliminate the multiplicity of names, in 1986 the International Committee on Taxonomy of Viruses recommended that the retrovirus identified should be re-named as HIV.

The first HIV therapy appeared in 1987, when a clinical trial showed that azidothymidine (AZT) decreased mortality and opportunistic infections in patients with AIDS [49]. However, viral resistance was quickly developed, and new drugs had to be developed [50]. It was not until 1996 that a combination of a protease inhibitor plus two nucleoside reverse transcriptase inhibitors markedly reduced the AIDS morbidity

and mortality [51]. This effective treatment was called highly active antiretroviral treatment (HAART). Unfortunately, although HAART provides viral control, HIV has a built-in survival mechanism, creating reservoirs of latent virus that are invisible to both HAART and the immune system. Furthermore, in spite of antiretroviral treatment, HIV infection is associated with abnormal chronic inflammation, and immune activation, which might result in accelerated immunosenescence [52].

3.2. HIV classification

HIV belongs to the group VI of reverse transcribing viruses, *Retroviridae* family, *Orthoretrovirinae* subfamily, and *Lentivirus* genus. It includes the HIV-1 and HIV-2 species, which share many similarities, including basic gene arrangement, mode of transmission, and clinical consequences [53]. However, HIV-2 is characterized by lower transmissibility and reduced progression to AIDS. From an epidemiological point of view, HIV-2 remains largely confined to West Africa, whereas HIV-1 extends worldwide [54]. 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: A, B, C, D, F, G, H, J, K, and Circulating Recombinant Forms (CRF).

3.3. HIV-1 morphology

HIV-1 matured virions are spherical particles of approximately 145 nm of diameter. The outer part of the virus is composed of a lipid bilayer derived from the host cell during the budding of newly formed virions (**Figure 2**). This lipid bilayer exposes the envelope glycoprotein (Env; gp160) consisting of 3 molecules of gp120 anchored to the membrane by the gp41 transmembrane protein [55]. It also contains several cellular membrane proteins derived from the host cell membrane, including MHC molecules, adhesion molecules, such as leukocyte function-associated antigen (LFA-1) and intercellular adhesion molecules (ICAMs), and co-stimulatory molecules such as CD80, CD86, etc. [56]. Several matrix proteins (MA; p17) lie in the inner surface of the viral membrane, and a conical capsid core (CA; p24) is located in the center of the virus. The capsid encapsulates two copies of the unspliced viral genome, which are stabilized as a ribonucleoprotein complex along with the nucleocapsid protein (NC; p7). Virus

particles also contain: protease (PR), reverse transcriptase (RT) and integrase (IN) enzymes; accessory proteins: Nef, Vif and Vpr; and three additional accessory proteins: Rev, Tat and Vpu (as reviewed by [55, 57, 58] (**Figure 2**).

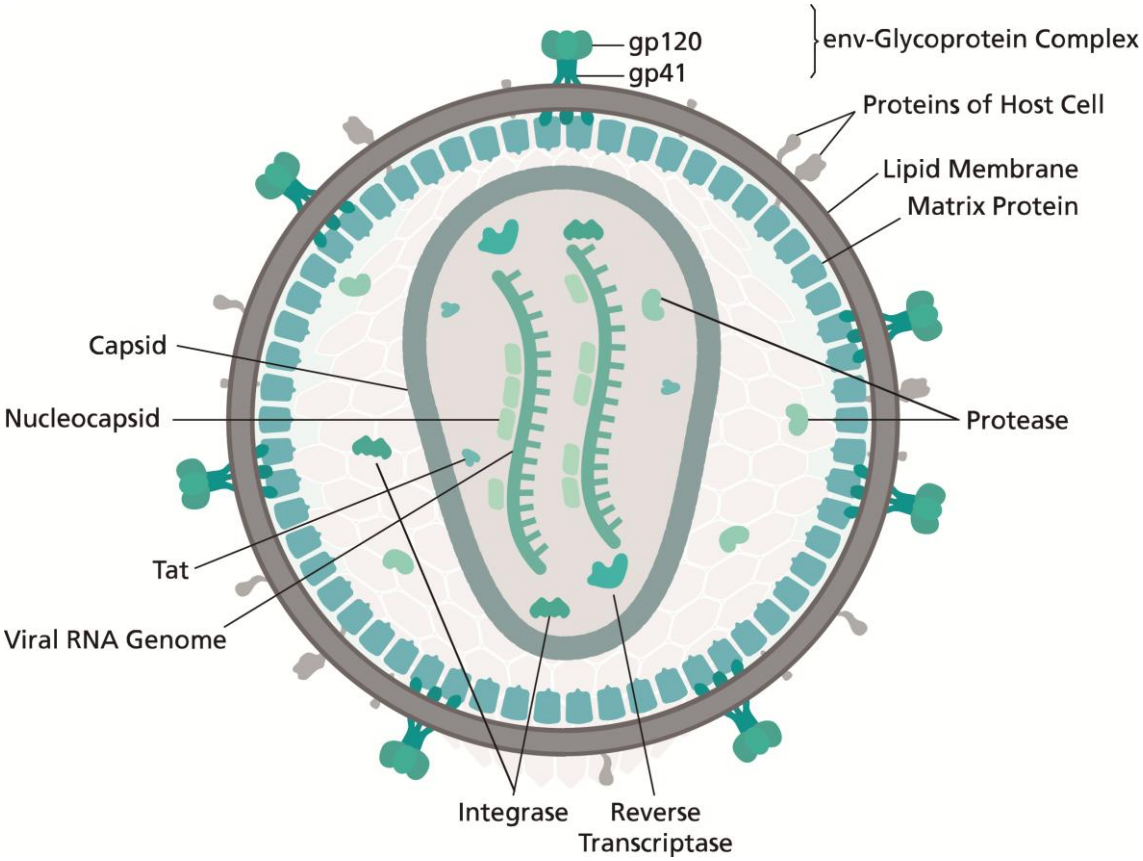


Figure 2. Schematic representation of the structure of HIV. Adapted from "HIV-structure en" by Thomas Splettstoesser (www.scistyle.com) - Own work. Licensed under CC BY-SA 4.0 via Commons - https://commons.wikimedia.org/wiki/File:HI-virion-structure_en.svg#/media/File:HI-virion-structure_en.svg

Each viral protein acts sequentially and has an important role in allowing viral replication, mediating all the steps involved between viral entry into the host cell until the budding and maturation of the new virion.

3.4 HIV-1 replication cycle

HIV-1 uses the machinery of the target cells to multiply and spread throughout the body. This process, which includes several steps, is called the HIV-1 replication cycle or HIV-1 life cycle.

a) Viral entry: It is a complex and intricate process that facilitates delivery of the viral genome to the host cell (**Figure 3A**). The main viral surface protein implicated is Env,

which is composed of a trimer of gp120 and gp41 heterodimers. First, the virion binds to the host cell, which can be facilitated by distinct cell attachment factors, allowing gp120 Env subunit to bind the host protein receptor CD4. CD4 binding induces conformational changes in Env that enables engagement of a chemokine coreceptor, such as CCR5 or CXCR4, and triggers membrane fusion. Finally, the gp41 subunit of Env enables fusion of the viral and host membranes allowing delivery of the viral cargo [57] (**Figure 3A**).

b) Reverse transcription: Membrane fusion leads to uncoating of viral capside delivering replication enzymes Reverse Transcriptase, Integrase, as well as the viral genomic RNA to the cytoplasm of the cell (**Figure 3A**). RT retro-transcribes the RNA into a double-stranded linear DNA that complexes with other proteins, forming the pre-integration complex. This pre-integration complex is translocated to the nucleus where the viral DNA is inserted into the host genome by the integrase activity [55, 59, 60]. Depending on the activation status of the target cells, the integrated viral DNA can remain latent in the host genome, or otherwise, it can proceed to the transcription and translation step.

c) Transcription and translation: After integration into the host genome, HIV-1 produces sequentially different mRNAs which encode for the viral proteins and the virion genomic RNA: early transcripts encoding Tat and Rev, which control the transcription process; then Env and the HIV-1 accessory genes Vif, Vpr and Vpu; and late-transcripts that encode the virion genomic RNA and the mRNA for the Gag-Pol polyprotein are synthesized. Rev transports the mRNAs encoding the structural proteins from the nucleus to the cytoplasm, where they are translated [61] (**Figure 3B**).

d) Viral assembly, budding and maturation: These events all appear to occur simultaneously at the plasma membrane, where Gag couples membrane binding, virion assembly, and RNA packaging (**Figure 3B**). HIV-1 virion assembly occurs at the plasma membrane, within specialized cholesterol-enriched micro-domains [62] and share glycosphingolipids and various membrane proteins that reside in lipid rafts [63]. The Gag polyprotein initially assembles into spherical immature particles, in which the membrane-bound Gag molecules project radially towards the interior of the virion. As the immature virion buds, protease is activated and cleaves Gag into its constituent

matrix, capsid, nucleocapsid, and p6 proteins. Proteolysis is required for conversion of the immature virion into its mature infectious form [64]. Although Gag itself can bind membrane, and assemble into spherical particles, the budding event that releases the virion from the plasma membrane is mediated by the host endosomal sorting complexes required for transport (ESCRT) machinery. Importantly, Viral Like Particles (VLPs) containing only the Gag protein fused to a reporter protein, such as Green Fluorescence Protein (GFP), can be produced and easily released to use them in viral capture assays.

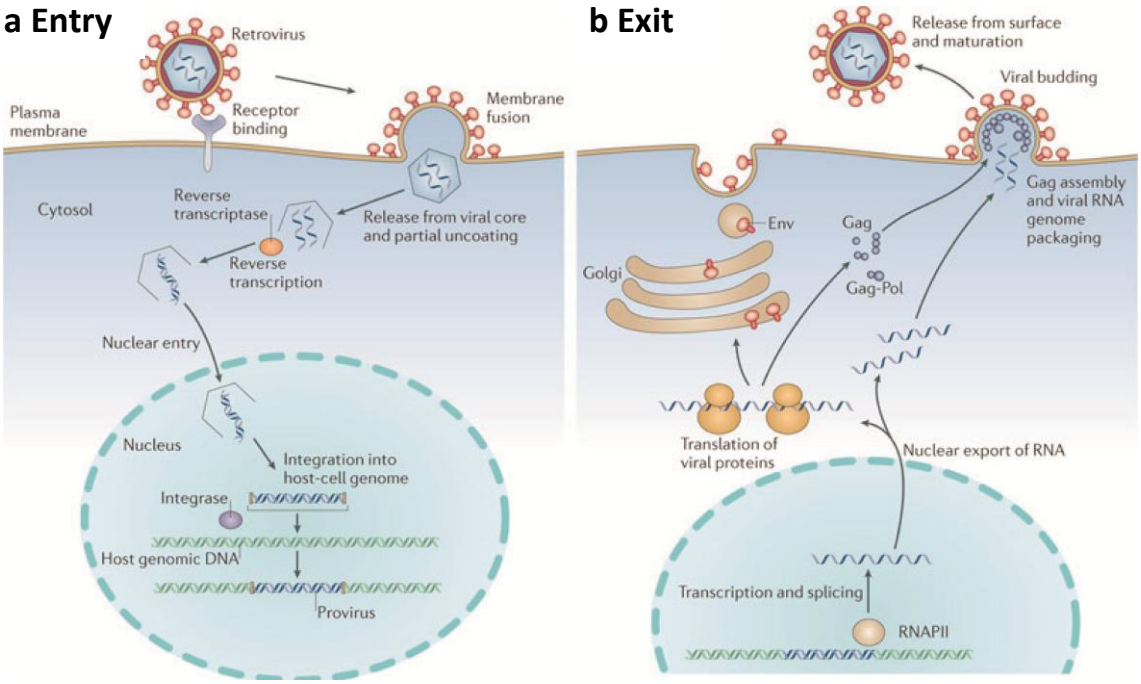


Figure 3. Schematic representation of the general HIV replication life cycle. A. Viral entry into cells involves the following steps: binding to a specific receptor on the cell surface; membrane fusion either at the plasma membrane or from endosomes (not shown); release of the viral core and partial uncoating; reverse transcription; transit through the cytoplasm and nuclear entry; and integration into cellular DNA to generate a provirus. **B.** Viral exit involves the following steps: transcription by RNA polymerase II (RNAPII); splicing and nuclear export of viral RNA; translation of viral proteins, Gag assembly and RNA packaging; budding through the cell membrane; and release from the cell surface, and virus maturation. Figure from [65].

HIV-1 mainly infects cells of the immune system, preferentially $CD4^+$ T lymphocytes, but also monocytes, macrophages, and DCs. HIV-1 infection course is characterized by a gradual decline of $CD4^+$ T cells, chronic immune activation, and the impairment of innate and adaptive immune responses that will eventually end in AIDS and lastly,

death. The natural course of untreated HIV-1 infection is characterized by a period that lasts an average of 11 years before progression to AIDS [66]. Once HIV-1 is transmitted, HIV-1 infection course can be divided within 4 stages: eclipse phase, acute or primary infection, chronic infection, and AIDS.

3.5. Transmission and stages of HIV-1 infection

HIV-1 can be transmitted via the exchange of a variety of body fluids from infected individuals, such as blood, breast milk, semen and vaginal secretions. These body fluids must come into contact with a mucosal membrane or damaged tissue or be directly injected into bloodstream (by a needle or syringe) for transmission to occur. The vast majority of HIV-1 infections result from mucosal transmission, with heterosexual transmission being the main route of viral dissemination accounting for 80% of HIV-1 infections worldwide [67]. Clinically, we could distinguish the following phases in HIV-1 untreated infection:

a) Eclipse phase (1–2 wk): Cell-free and cell-associated virions cross genital mucosa barrier and infect susceptible target cells, such as $CD4^+$ T cells, macrophages, Langerhans cells and myeloid DCs, which reside within the epithelium or lamina propia [68]. HIV-1 can then spread from the site of infection to other parts of the body by entering the blood and lymphatic vessels in the mucous membrane tissue. The virus must not only establish a small founder population of infected cells at the portal of entry, but also expand that local infection to continue disseminating the virus and the newly infected cells via lymphatic drainage. This establishes a self-propagating infection in the genital draining lymph nodes [69][70]. Throughout this phase, viremia is undetectable, and neither immune response nor symptoms of infection are yet visible (**Figure 4**).

b) Acute (or primary) infection (2–4 wk): infection spreads to establish systemic infection throughout the secondary lymphatic tissues: spleen, gut-associated lymphatic tissue (GALT), and peripheral lymph nodes [69][70]. Acute phase is characterized by high levels of viremia (up to 10^7 or more copies of viral RNA per milliliter of blood), and the presence of large proportions of infected $CD4^+$ T cells in GALT, blood and lymph nodes. This phase is often, but not always, accompanied by

"flu-like" symptoms—fever, enlarged lymph nodes, throat inflammation, etc. Around the time of peak viremia, the immune response starts with antibodies against viral proteins and with $CD8^+$ T -cell responses against HIV-1 antigens expressed on infected cells. At the end of the acute phase, the level of viremia declines sharply, 100-fold or more, as a result of partial control by the immune system and the exhaustion of activated target cells. This phase is also characterized by a transient decline in the numbers of $CD4^+$ T cells in blood (**Figure 4**).

c) Chronic infection (1–10 yr): Chronic infection, or "clinical latency," is characterized by a constant increase in viremia, usually in the order of 1–100,000 copies/mL, referred as the viral load "set point," and a steady, close to normal, or gradually falling level of $CD4^+$ T cells. Patients in this phase are asymptomatic and usually unaware that they have been infected (**Figure 4**).

d) AIDS: The number of $CD4^+$ T cells declines to the point where immune control is no longer maintained (<200 cells/μL), and opportunistic infections appear. Immune control over HIV-1 is also lost, and the level of viremia rises, culminating in the death of infected individuals. Indeed, untreated HIV-1 infection is one of the most lethal infectious diseases known, with a mortality rate over 95% [60] (**Figure 4**).

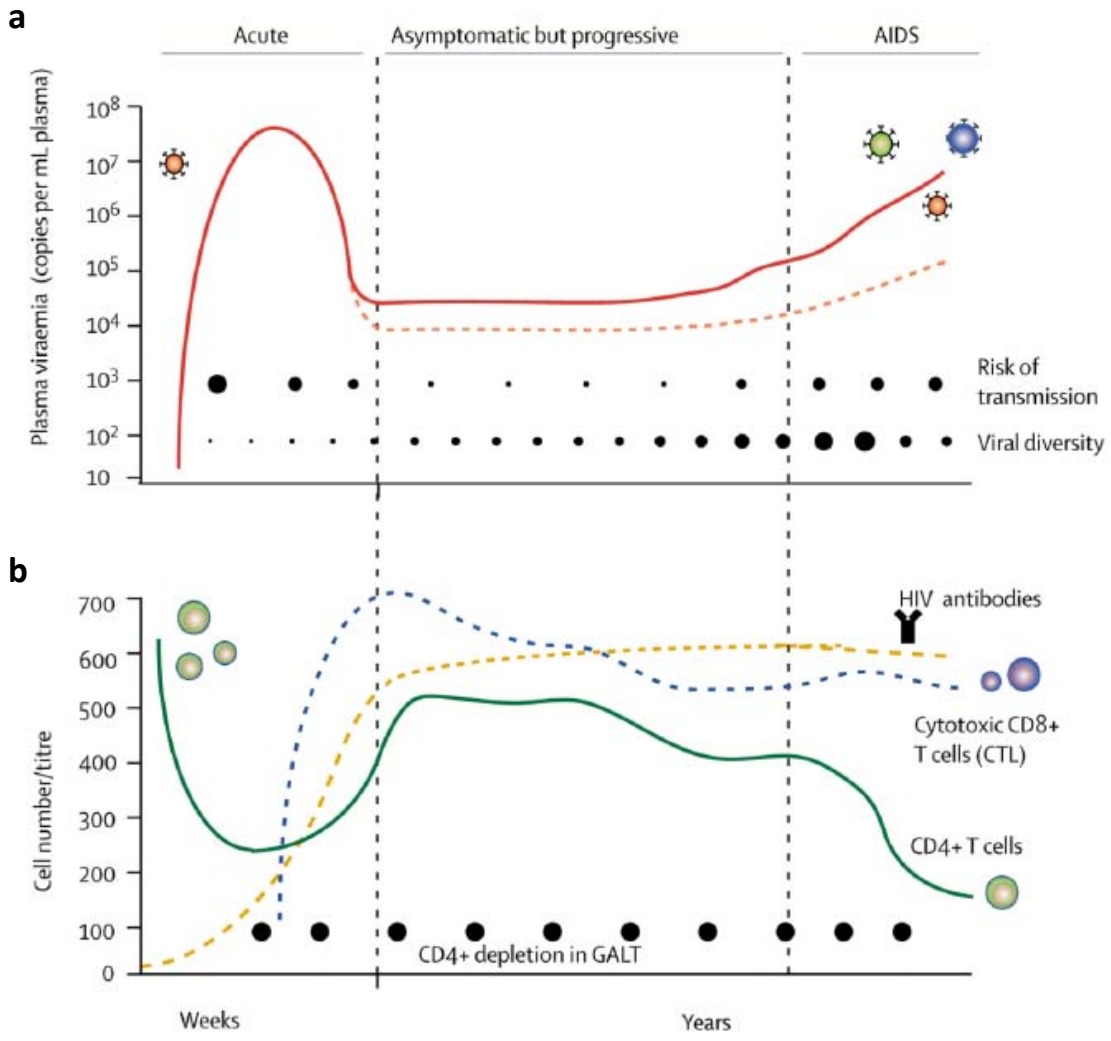


Figure 4. Course of HIV-1 infection. A. Plasma viremia and **B.** dynamic changes of lymphocytes and produced specific antibodies. Acute infection is characterized by high plasma viremia (red line), low $CD4^+$ T cell count (green line) and absence of HIV-1 specific antibodies (yellow line). Viremia drops as cytotoxic $CD8^+$ T-lymphocytes develop (blue line) and an individual viral-load set point is reached during chronic infection. It follows an asymptomatic but progressive phase or also called chronic infection characterized by stable high viral load, a gradual depletion of $CD4^+$ T cells and inefficient HIV antibody production and cytotoxic $CD8^+$ T cell responses. AIDS is characterized by an uncontrolled viremia and a decline in $CD4^+$ T cell count to below 200 cells/µl. Viral diversity increases throughout the disease (closed circles, **A**). The risk of transmission is highest in the first weeks when viremia peaks and during AIDS phase (closed circles, **A**). $CD4^+$ T cell depletion in GALT occurs during acute phase and is maintained throughout the HIV infection course. GALT=gut-associated lymphoid tissues. Figure from (Simon, Ho, and Abdool Karim 2006).

Thus, infection with HIV-1 results in prolonged, continuous viral replication within the infected host. Remarkably, viral persistence is not prevented by the presence of apparently vigorous virus-specific immune responses. Introduction of successful antiretroviral treatment results in viremia suppression, $CD4^+$ T cell recovery, and

reduction in systemic immune activation. Despite lowering morbidity and mortality, therapy must continue throughout life, and virus rapidly rebounds if HAART is stopped. Several factors are thought to contribute to persistent viral replication, most notably the destruction of virus-specific T helper cells, the emergence of antigenic escape variants, the antibody inaccessibility to the envelope protein, the impairment of antigen presentation, and the integration and latency of HIV-1 genome into anatomical reservoirs [71].

In addition, HIV-1 evades the immune system by exploiting the myeloid cell function. Myeloid cells contribute to viral pathogenesis through *trans*-infection, a mechanism that promotes viral capture and transmission to target cells, which become effectively infected [72]. Defining the interactions between HIV-1 and myeloid cells and understanding the role of antigen presenting cells during the course of HIV-1 infection are thus essential to fight against HIV-1.

4. HIV-1 Interactions with Myeloid Cells

Myeloid cells are one of the first cells to encounter the virus, and the specific interaction that occurs between these cells and HIV-1 is critical for HIV-1 to establish infection. Most importantly, HIV-1 is able to efficiently transfer the virus to its primary target cell, the $CD4^+$ T lymphocyte, in which it replicates explosively.

Based on viral source, we can distinguish two types of HIV-1 interactions with myeloid cells: cell-free virus infection or cell-mediated transmission. Cell-free virus infection is produced by free-floating viruses that are able to directly infect myeloid cells. Cell-mediated transmission takes place when myeloid cells (donor cells) transfer virus to uninfected $CD4^+$ T cells (receptor cells). This cell-to-cell viral transmission to $CD4^+$ T cells can be mediated through virological synapses, where donor myeloid cells are productively infected, or through infectious synapses, where donor myeloid cells are not productively infected but harbor the virus within the cell (**Figure 5**).

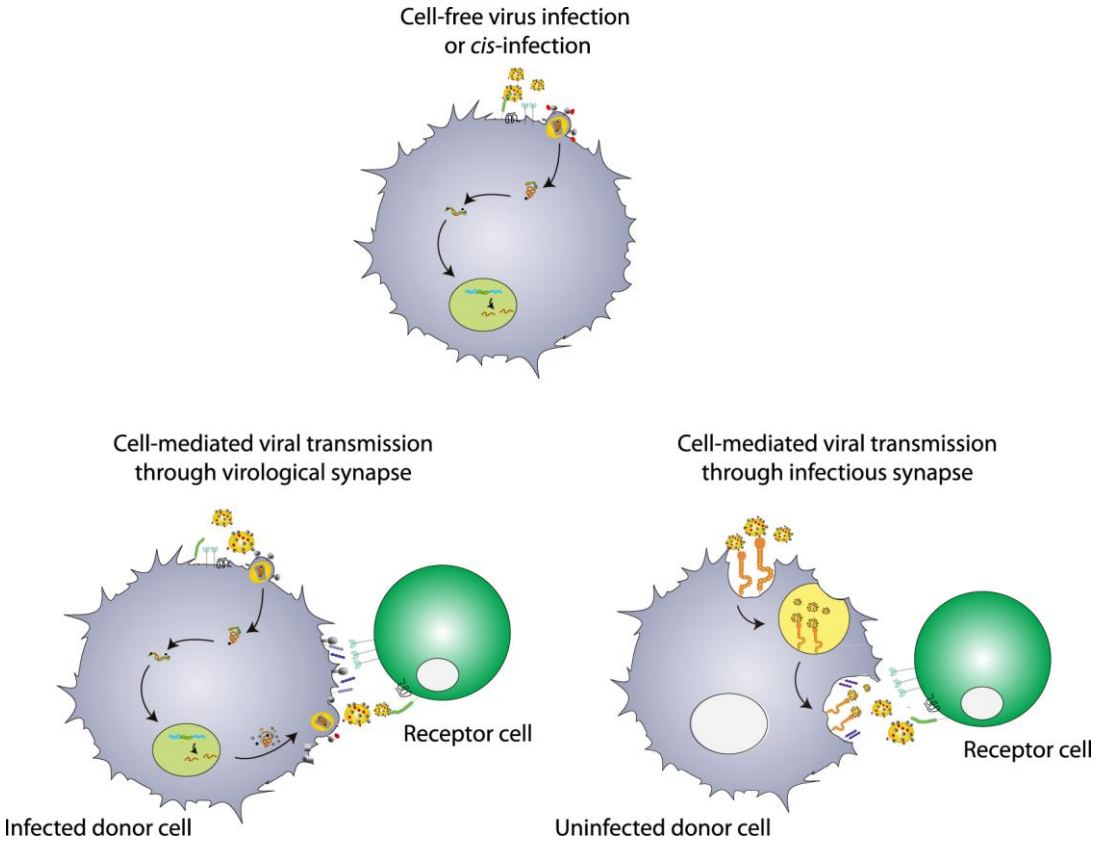


Figure 5. Different HIV-1-myeloid cell interactions: cell-free virus infection and cell-mediated viral transmission. Cell-mediated viral transmission can be mediated through virological synapses (donor cells are infected), or through infectious synapses (donor cells are not infected).

4.1. Cell-free virus infection or *cis*- infection

Although $CD4^+$ T cells are the main HIV-1 targets, myeloid cells are also susceptible to HIV-1 infection. Studying HIV-1 infection of myeloid cells *in vivo* is complex, owing to the low frequency of these cells in HIV-1 infection, their constant migration from mucosal to lymphoid tissues, and the difficulty to obtain cells from these tissues [41].

Cell-free HIV-1 binds myeloid cells via additional specific receptors other than CD4. The C-type lectin receptors (CLRs), such as the dendritic cell-specific intercellular adhesion molecule-3 (ICAM)-grabbing non-integrin (DC-SIGN or CD209) (**Figure 5**) [73, 74] and dendritic cell immunoreceptor (DCIR) [75], interact with virus particles by binding high-mannose oligosaccharides on the heavily glycosylated HIV-1 Env [76–78]. While DCIR is expressed on all myeloid cells, DC-SIGN is expressed only on DCs and macrophages. HIV-1 Env can also bind to immature DCs by interacting with the charged residues of

heparin sulfate proteoglycans (HSPG) [79] or Syndecan-3 [80]. These mechanisms of HIV-1 binding in myeloid cells facilitate viral interactions with CD4 and viral fusion events leading to *cis*- infection.

All myeloid cells express CD4 and the chemokine co-receptors CCR5 or CXCR4 rendering them susceptible to infection with HIV-1 [81–83]. However, cell-free HIV-1 infection in myeloid cells is generally less productive compared to $CD4^+$ T cells [41]. Limited productive viral infection in myeloid cells could be attributed to lower expression of CD4, CCR5 and CXCR4 [81], and to the internalization of HIV-1 trough phagocytic processes, leading to rapid and extensive viral degradation [73, 84]. R5 HIV-1 strains can replicate more efficiently than X4 HIV-1 strains in myeloid cells, being a possible strainer of viral tropism during HIV-1 transmission. HIV-1 infection permissiveness of myeloid cells can vary greatly, depending on their tissue localization, cytokines surrounding, and other environmental factors. For example, maturation of DCs is associated with a marked decline in HIV-1 fusion [85], leading to a decreased in HIV-1 replication in mature DCs, compared to immature DCs [83, 86]. As regards monocytes, it appears that a non-classical monocyte population $CD14^+$ $CD16^{++}$, which are expanded in HIV-1 infection, are more susceptible to HIV-1 infection and preferentially harbor the virus for long periods, compared to $CD14^{++}$ $CD16^-$ classical monocytes [87, 88]. While sub-epithelial macrophages from vaginal mucosa are susceptible to HIV-1 infection due to high expression of CCR5 co-receptor, jejunum intestinal macrophages are resistant to HIV-1 infection because they do not express CCR5. Infected macrophages have a low but constant viral replication capacity, emphasizing the importance of this viral source.

Viral infectiveness in myeloid cells is also modulated by the expression of host restriction factors, such as tetherin [89], apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) [90] and SAM domain and HD domain containing protein 1 (SAMHD1) [91], which represent important innate immune mechanisms against retroviral infection [92].

In contrast to cell-free virus infection, cell-to-cell viral transmission has been shown to be a more rapid and efficient mechanism, where virus and viral receptors are found concentrated at the cell-to-cell synapse [93]. Thus, mathematical models predict that

only ~ 10% of the infections in the lymphoid tissue are spread by cell-free virions, while the remaining ~ 90% are transmitted by cell-to-cell transmission [94].

4.2. Cell-mediated viral transmission via virological synapse

Despite being less effective than HIV-1 infection of $CD4^+$ T cells, HIV-1 can infect myeloid cells that could produce progeny virions that are released to infect new target cells via the close contact and formation of a virological synapse (**Figure 6A**). The primary signal for assembly of virological synapse between an infected donor and an uninfected target cell, is Env-CD4 binding. Subsequent stabilization is achieved by interactions between ICAM-1, ICAM-3 and LFA-1 [95]. Finally, the virus buds at the interface and infects the target cell in a CD4-dependent manner [96–98].

Cell-mediated viral transmission that requires *de novo* synthesis of new virus has been explained in immature DCs as a delayed phase that occurs for 1 to 2 days upon viral exposure [73]. Although there is limited published data about viral transmission by HIV-1-infected monocytes, circulating monocytes harboring HIV-1 provirus that migrate and differentiate to macrophages could promote HIV-1 replication and cell-mediated viral transmission to uninfected cells [99]. HIV-1 replication in macrophages is characterized by the assembly and storage of viral particles in internal cytoplasmic vacuoles [100]. These viral compartments are non-classical endosomal compartments that allow the budding virus to traffic to uninfected $CD4^+$ T cells, mediating viral transmission through infected cells to uninfected cells via virological synapses [101].

4.3. Cell-mediated viral transmission via Infectious synapse

Another cell-mediated viral transmission consists in that virus retained at or near the cell surface is transmitted to a target cell through infectious synapses, a structure analogous to the immunological synapse [102, 103].

Unlike the virological synapse, the infectious synapse does not rely on the productive infection of the donor cell, but also allows for viral transmission to target $CD4^+$ T cells [104] (**Figure 6B**). The process of viral transmission via formation of infectious synapse is called *trans*-infection. HIV-1 *trans*-infection has been mostly studied in DCs [105],

but other myeloid cells such as monocytes [106] have the potential to establish infectious synapses with target cells [106].

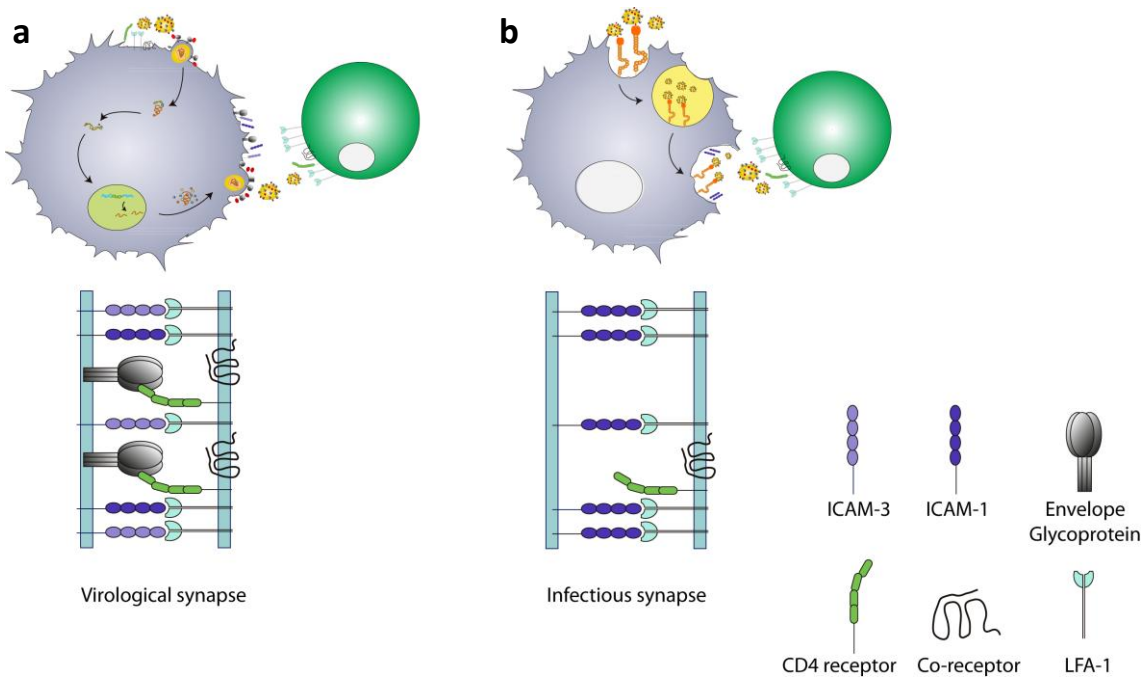


Figure 6. Comparison between cell-mediated viral transmission through A. virological synapse and B. infectious synapse (also called *trans*-infection) by DCs. A. In the virological synapse, the primary signal for assembly between an infected donor and an uninfected target cell, is Env-CD4 binding. Subsequent stabilization is achieved by interactions between ICAM-1, ICAM-3 and LFA-1. Finally, the virus buds at the interface and infects the target cell in a CD4-dependent manner. **B.** In the infectious synapse, first, adhesion molecules ICAM-1 and LFA-1 participate in the formation of uninfected DC-CD4$^+$ T-cell conjugates. Then, there is a recruitment of HIV-1 CD4 receptor and CXCR4 and CCR5 co-receptors on the T cell, forming the infectious synapse. Finally, the virus storage compartment in DCs is shifted towards this contact zone, facilitating rapid and efficient infection of the neighboring T cell.

The *trans*-infection mechanism involves uptake of HIV-1, storage of viruses in a non-degrading compartment, and final release of the virions at the cell to cell contact zone, which enables infection of the target cell [77, 103, 107, 108]. Direct visualization using GFP-tagged HIV-1 revealed that DCs *trans*-infect HIV-1 by first binding and then concentrating the intact virus at the cellular surface of the donor cell [108, 109]. This viral accumulation leads to the formation of a non-classical endosomal, non-acidic compartment enriched in tetraspanins and MHC class II, which can be under constant remodeling over time [110]. Adhesion molecules, such as ICAM-1 and LFA-1, participate first in the formation of DC-CD4$^+$ T-cell conjugates [102, 111]. Then, there is

a recruitment of HIV-1 CD4 receptor and CXCR4 and CCR5 co-receptors on the T cell, forming the infectious synapse [103]. Finally, the virus storage compartment in DCs is shifted towards this contact zone, facilitating rapid and efficient infection of the neighboring T cell [103]. 3D electron microscopy images of virological synapses showed that membrane extensions are originated from uninfected $CD4^+$ T cells, either as membrane sheets or as filopodial bridges, accessing viral compartments and being involved in HIV-1 transmission from DCs harboring virus to uninfected $CD4^+$ T cells [112].

Although this non-conventional compartment has been mostly studied in DCs, an interesting similar structure exists in HIV-1-infected macrophages [113, 114]. These virus-containing compartments in infected macrophages were initially thought to be late endosomes or multivesicular bodies, but it has been shown that they are separated from the endocytic pathway, co-localize with several tetraspanins (CD63, CD53, CD9, CD81 and CD82), adhesion molecules, MHC classe II and present a non-acidic pH, similar to the DC storage compartment [114, 115]. This compartment also has the ability to allow virus trafficking to a T-cell contact zone, thus facilitating cell-to-cell viral transfer via virological synapse, suggesting that this compartment targeted by HIV-1 is similar in macrophages and DCs. The main difference between the systems is that in macrophages virus undergoes assembly on this compartment, while in DCs it gains access through endocytosis [116].

In both cell types, this virus-containing compartment is connected to the extracellular milieu, and is accessible to small membrane-impermeable dyes such as ruthenium red, horseradish peroxidase and lucifer yellow [100, 109, 113, 117]. However, high molecular weight substances, including broadly neutralizing antibodies are excluded [118, 119]. Thus, infectious HIV-1 retained within these compartments in macrophages and DCs seem to be protected from neutralizing antibodies [120]. In sum, this virus-containing compartment represents a hideout for HIV-1 in myeloid cells, that serves both as an HIV-1 reservoir and as a potent disseminator of the virus within tissues [118, 121].

The precise nature and origin of the capture and storage compartment is currently unknown. It has been demonstrated that capture and transfer of HIV-1 by mature DCs

converges with a pre-existing traffic pathway of exosomes, which are small secreted vesicles bearing antigens that traffic between APCs [122]. Exosome trafficking augments antigenic presentation and amplifies the immune response [122]. Thus, HIV-1 and exosomes compete for DC capture, indicating that they utilize the same pathway [123]. Therefore, HIV-1 is exploiting a pre-existing immune pathway to spread its infection to new target cells.

5. Identification of Siglec-1 as the HIV-1 receptor implicated in *trans*-infection

The mechanism by which DC capture HIV-1 and promote *trans*-infection of $CD4^+$ T cells has been topic of debate over the last decade. The initial Trojan horse hypothesis suggested that HIV-1 captured by immature DCs in the mucosa may protect the virus from degradation and allow its transport to secondary lymphoid organs, facilitating *trans*-infection of $CD4^+$ T cells and viral spread [104, 124, 125]. DC-SIGN expressed on immature DCs was described as the main receptor responsible for viral capture and *trans*-infection of uninfected $CD4^+$ T cells (**Figure 7A**) [104]. However, subsequent reports demonstrated that most of the captured virions by DC-SIGN are rapidly degraded and presented via MHC class I and MHC class II to T cells, eliciting adaptive immune responses [126, 127]. The limited capacity of immature DCs to sustain *trans*-infection [73] and the limited contribution of DC-SIGN to viral transmission was argued in several independent studies [126, 128–131].

In 2009, viral lipid composition was shown to be involved in viral capture by mDCs. HIV-1 particles produced from cells where glycosphingolipid (GSL) synthesis was inhibited, produced virions with impaired capture and transfer [123]. Furthermore, by modifying the lipid composition of liposomes that mimic the size of HIV-1, but lack any proteins, it was discovered that gangliosides, which are glycosphingolipids with one or more sialic acids **(Figure 7B)**, are involved in viral capture [132]. Specifically, the sialyllactose molecule present in some gangliosides was identified as the determinant moiety for mDC HIV-1 uptake (**Figure 7C**) [132].

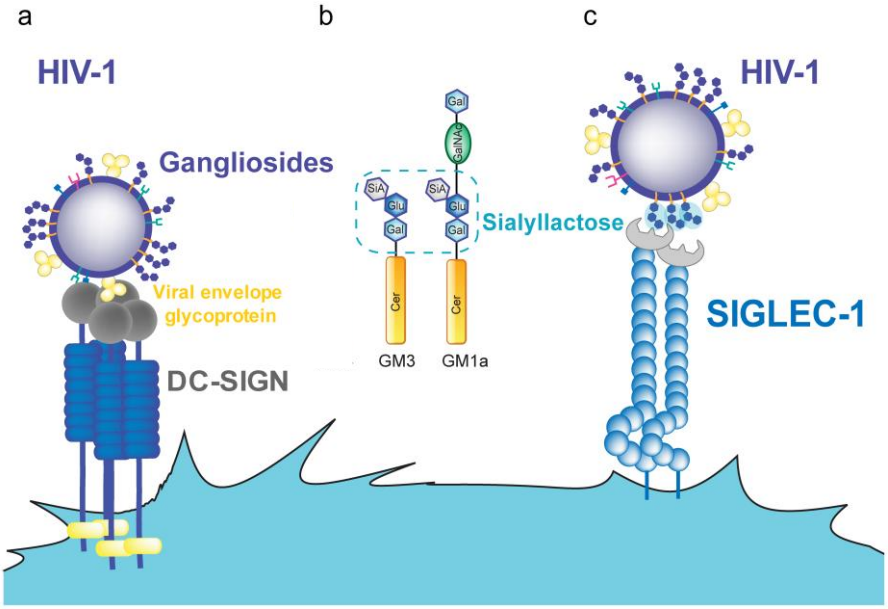


Figure 7. HIV-1 binding to DC receptors. A. HIV-1 can bind to DC-SIGN via recognition of the viral envelope glycoprotein. **B.** Several gangliosides in the HIV-1 lipid membrane expose a sialyllactose moiety. **C.** Siglec-1 can capture HIV-1 through recognition of sialyllactose moieties of viral membrane gangliosides. Abbreviations: Cer (ceramide), Gal (galactose), GalNAc (Nacetylgalactosamine), Glu (glucose), SiA (sialic acid). Figure from [133].

Once it was discovered that the sialyllactose motif from viral membrane gangliosides is the attachment factor that mediates viral capture by mature DCs, it was much easier to identify the attachment receptor present on DCs. The main candidates under study were the Siglec family, because these type I transmembrane proteins have an amino-terminal V-set domain that interacts with sialylated ligands [134]. Among all candidates, Siglec-1 was identified as the key factor for HIV-1 spread via infectious DC/T-cell synapses, highlighting a novel mechanism responsible for HIV-1 dissemination (**Figure 7C**) [135], which was later confirmed in an independent publication [136]. Siglec-1 structure, function and its role as a pathogen receptor, is summarized below.

5.1 Siglec-1 has a unique structure that favors *trans*-infection

Siglec-1, also known as Sialoadhesin or CD169, is a type I lectin transmembrane glycoprotein that belongs to the Ig superfamily. It consists of an unusually large extracellular region of 17 domains, comprising an N-terminal V-set domain that contains the sialic acid binding site followed by 16 Ig-like C2-set domains [137, 138] (**Figure 8A**). Siglec-1 has an unusual pattern of conserved cysteine residues characteristic of the Siglec family that gives rise to an intrasheet disulfide bond within domain 1 and a disulfide bond between domains 1 and 2 [134]. Human Siglec-1 is a highly conserved protein within mammals, which is 72% identical to murine Siglec-1. Alignment of the N-terminal region of human and mouse Siglec-1 showed that amino acids important for sialic acid binding are identical, as well as the pattern of cysteine residues [134, 137].

Siglec-1 specifically recognizes *N*-acetylneuraminic acid (Neu5Ac, one of the most common sialic acids in mammals) linked at α2–3Gal in N- and O-glycans (**Figure 8B**). Studies that investigated the interaction between Siglec-1 and Neu5Ac, demonstrated by site-directed mutagenesis [139], X-ray crystallography [134], and nuclear magnetic resonance (NMR) [138] that amino acids important for sialic acid binding include an arginine residue at position 116 (which is conserved in all species) and two conserved tryptophans at positions 21 and 125. Use of synthetic sialosides revealed significant but low affinity, for monovalent ligands (k_D in the range of 1 mM). Thus, most likely, simultaneous multivalent low affinity associations create sufficient high avidity and lead to biologically meaningful interactions of Siglec-1 for sialic acids on cells or pathogens [140].

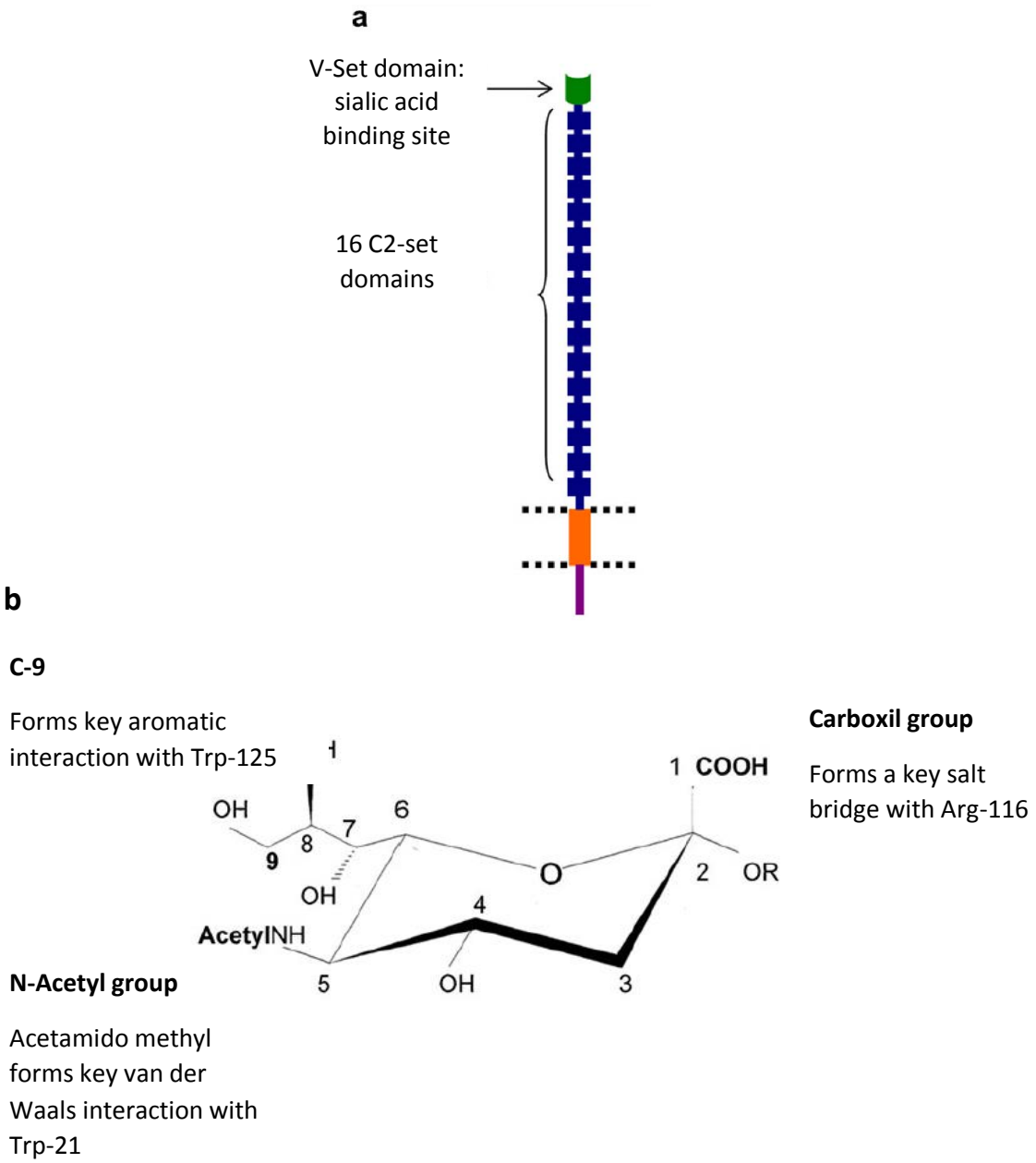


Figure 8. The features of Siglec-1. A. Schematic diagram showing the domain organization of Siglec-1 protein. **B.** N-acetylneuraminic acid showing the functional groups that make key contacts with the binding site of Siglec-1. Figure adapted from [141].

Because Siglec-1 possesses a long extracellular domain that can extend the ligand-binding site far from the cell-surface preventing Siglec-1 binding to sialic acids from the same cell (*cis*- binding), it is suggested that Siglec-1 mediates cell–cell interactions [137]. Furthermore, in contrast to the majority of other Siglecs, Siglec-1 does not contain any inhibitory tyrosine-based motifs in its relatively short cytoplasmic tail, suggesting a primary role as binding partner in cell-to-cell interactions rather than in

cell signaling [120, 142]. The long extended Siglec-1 protein structure also can mediate recognition and uptake of sialic acid covered pathogens. Thus, Siglec-1 protrudes outside the cell glycocalyx and can recognize sialyllactose motifs exposed on the HIV-1 membrane.

5.2. Siglec-1 is an inducible receptor

Human Siglec-1 is constitutively absent from monocytes and other peripheral blood leukocytes, but expressed by tissue macrophages in the spleen, lymph node, bone marrow, liver, colon, and lungs [143]. However, Siglec-1 was also found on inflammatory monocytes and macrophages during chronic inflammation, as occurs during autoimmune diseases or viral infections [143–146], where type I IFN levels are up-regulated. That is the case of HIV-1 infection [145], systemic lupus erythematosus [147] and systemic sclerosis [144]. Although there is no *in vivo* evidence of Siglec-1 expression on DC, it can be induced *in vitro* after treatment of blood myDC and monocyte-derived DC with inactivated human rhinovirus [148] or after LPS or type I IFN stimulation [135, 136].

The regulation of Siglec-1 is broadly inflammatory and particularly subjected to type I IFN induction [136, 144]. Siglec-1 induction on human PBMCs and various macrophages subsets can be achieved by incubation with either type I IFN or TNFα [106, 146, 149]. Molecules that induce IFNα secretion, such as LPS and poly I:C throughout stimulation of TLRs involved in viral and bacterial sensing (via MyD88-independent pathway) also increase Siglec-1 expression [144]. Overall, this strongly suggests a function for Siglec-1 in anti-viral and anti-bacterial activities.

5.3. Siglec-1 in HIV-1 pathogenesis

HIV-1 infection results in a storm of different pro-inflammatory stimuli that might control and limit HIV-1 infection [150]. However, Siglec-1 induction by released IFNα could favor HIV-1 transmission in an otherwise antiviral environment. IFNα is a potent antiviral cytokine mainly produced by pDCs in response to HIV-1 challenge. IFNα-producing pDC represents the first line of immune defenses against viral infections [151–153]. Despite its antiviral effect, continuous pDC activation and IFN secretion

may result in a state of persistent immune activation that leads to exhaustion of the immune system, similar to that observed during chronic HIV-disease [151, 154].

Non-Human Primate (NHP) models of Simian Immunodeficiency Virus (SIV) infection have been used to study the role of immune activation in the pathogenesis of HIV-1, particularly through the comparison of NHP pathogenic models, that recapitulate human HIV-1 infection course, with non-pathogenic models. Pathogenic models are those in which the SIV-infected animals generally progress to AIDS, such as *Rhesus macaque* (**Figure 9C**). On the other hand, non-pathogenic models are those in which SIV infection of host animals does not appear to cause disease, such as SIV infection of *Sooty mangabey* (**Figure 9A and B**). While HIV-1 human infection and NHP pathogenic models of SIV infection are characterized for presenting a persistent immune activation, non-pathogenic models of SIV infection present a temporary acute immune activation independently of viral load. Thus, activation of the immune system is a hallmark of progressive HIV-1 infection and better predicts disease outcome than plasma viral load, yet its etiology remains obscure (as reviewed in [155]).

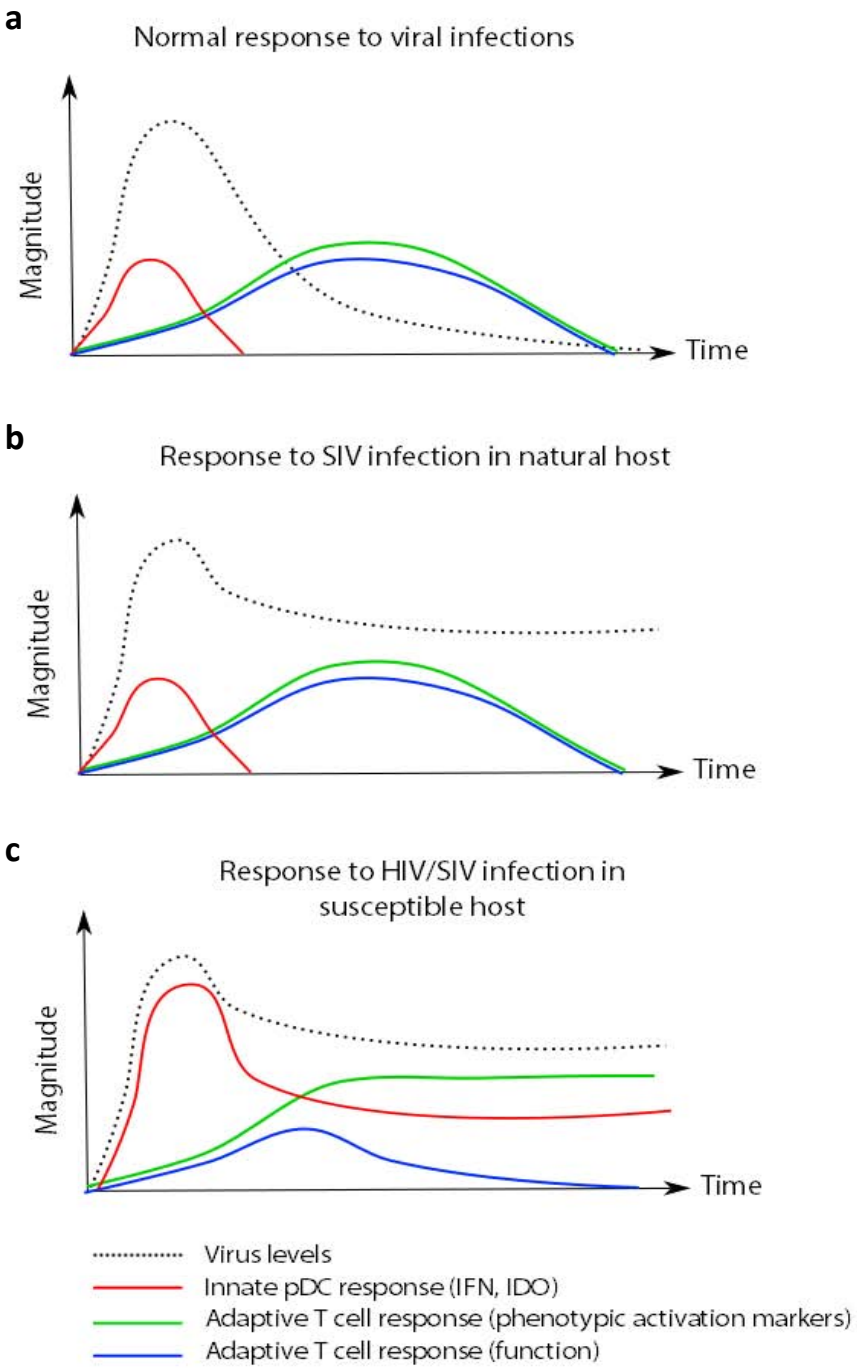


Figure 9. Chronic innate immune activation as a cause of chronic T cell activation and functional impairment. A. During most viral infections, early innate immune responses promote and are replaced by efficient adaptive T cell responses, ultimately resulting in the clearance or control of the infectious agent. **B.** HIV/SIV infection of natural resistant hosts is not efficiently cleared by immune responses, but the immune system appears to respond normally to the infection, and no signs of chronic immune activation or immune deficiency are observed. **C.** We hypothesize that HIV/SIV infection of susceptible hosts results in abnormal and prolonged activation of innate immune responses, resulting in: 1) induction of phenotypic markers of T cell activation; 2) progressive T cell depletion through apoptotic mechanisms; and 3) suppression of functional T cell responses by mechanisms such as IDO/T_{reg} or PDL-1. Figure adapted from [154]).

Since Siglec-1 is an inducible receptor, enhanced pro-inflammatory stimuli at different anatomical sites could induce Siglec-1 expression on myeloid cells, favoring viral spread, novel infections, and a chronic hyper-activation state of the immune system, which all lead to pathogenesis. As we will explain next, several key sites and relevant events throughout the course of HIV-1 infection could induce Siglec-1 expression and fuel disease progression.

The vast majority of new HIV-1 infections result from mucosal transmission [67]. Transmission events have been mainly studied in SIV-infected *Rhesus macaque*, a pathogenic non-human primate model that resembles to that of HIV-1 infection in humans (**Figure 9C**). On exposure to SIV, endocervical epithelial cells produce macrophage inflammatory protein 3 (MIP-3α), which attracts substantial numbers of pDCs to the mucosa [156]. pDCs are the first predominant cell type to arrive at infected mucosal sites and are activated by HIV-1 to produce pro-inflammatory cytokines, such as type I IFN [151]. Thus, resident myeloid cells exposed to these cytokines could up-regulate Siglec-1 expression and contribute to the amplification of viral infection at the mucosal sites. Furthermore, pDCs secrete MIP1β and other chemokines and cytokines that attract $CD4^+$ T cells, contributing to the establishment of a small founder viral population and to the burst of HIV-1 replication. Thereafter, HIV-1 spreads to local lymphoid tissues, and systematically to secondary lymphoid tissues, especially the gut-associated lymphoid tissue (GALT) [70].

GALT represents the largest immune compartment in the body. In fact, it has been estimated that more than 60% of all T cells may reside within the small intestine, emphasizing the potential contribution of the gut to systemic immunity [157]. The bulk of $CD4^+$ T-cell depletion occurs rapidly within the acute phase of HIV-1 infection, and results in a characteristic explosion of viremia. This massive $CD4^+$ T-cell depletion occurs predominantly in the gastrointestinal tract, where the integrity of gut mucosa is damaged and translocation of microbial products from the intestinal lumen to systemic circulation occur [158]. It has been found that plasma levels of LPS, a common structural component of bacteria and strong activator of innate immune responses, were increased in HIV-1-infected patients and SIV-infected macaques [150, 159, 160]. Some studies have found that LPS levels correlated with plasma IFNα,

increased expression of IFN-stimulated genes (ISGs), biomarkers of inflammation, and disease progression [150], indicating that microbial translocation is a cause of systemic immune activation [150].

Furthermore, it has been reported that there is a pDC redistribution from blood into the gut during acute SIV/HIV infection. pDCs up-regulate a gut-homing receptor CD103 and accumulate in jejunum, colon, and gut-draining lymph nodes of SIV-infected *Rhesus macaque* and HIV-1 infected individuals. Consequently, pro-inflammatory cytokine secretion by pDCs into the gut has been suggested to additionally contribute to persistent HIV-1 immune activation [157]. Whether this inflammatory milieu produced by microbial translocation and pDC homing to the gut mucosa, could induce Siglec-1 receptor on resident myeloid cells and contribute to viral spread needs further study (**Figure 10B**). Microbial translocation and a massive $CD4^+$ T-cell loss end in a persistent immune activation that begins during the acute phase of infection but is maintained during the chronic phase of HIV-1 infection [150].

During chronic stages of HIV-1 infection, there is a depletion of blood myDCs and pDCs that migrate and accumulate in secondary lymphoid tissues [161]. Interestingly, these cells display an altered maturation profile that resembles to that of IFNα activation [161]. Accumulated pDCs in lymphoid tissues are able to release large amounts of IFNα, which could contribute to the activated phenotype of resident myeloid cells [162]. Interestingly, IFN responses are sustained in pathogenic SIV models, while are only strongly induced during acute phase in non-pathogenic SIV infection models, but quickly resolved after peak viral load [163]. Noteworthy, Siglec-1 is up-regulated early after SIV infection in monocytes from both pathogenic and non-pathogenic models, but its expression is only maintained in pathogenic models [155]. Thus, sustained IFN responses could induce Siglec-1 expression on myeloid cells and enhance HIV-1 transmission to uninfected target cells in lymphoid tissues, facilitating HIV-1 progression. Furthermore, lymphoid tissues are sites where cell-to-cell encounters frequently occur; thus, Siglec-1-mediated viral transmission to bystander $CD4^+$ T cells could become the main viral transmission pathway in lymphoid tissues in chronic HIV-1 infection (**Figure 10C**).

Based on this information, we will evaluate how pathogenic and immunological factors such as IFNα modulate Siglec-1 expression on different myeloid cells and relevant human tissues. First, we will determine whether distinct myeloid cells have the capacity to up-regulate Siglec-1 in the presence of IFNα and mediate HIV-1 *trans*-infection *in vitro*. Furthermore, we aim to characterize the *ex vivo* pattern expression of Siglec-1 on myeloid cells located in blood and tissues, known to play a prominent role in HIV-1 disease progression, including those located in lymphoid areas. Our rationale is that, if Siglec-1 expression is detected *ex vivo* on these myeloid cells, this receptor could have an impact on HIV-1 transmission.

Our ultimate goal is to apply this knowledge to the design of a novel generation of therapeutic agents against Siglec-1, aimed at inhibiting the most potent HIV-1 cell-to-cell transmission mechanism known so far. Thus, we present a rational approach to screen between different inhibitory agents and select the most promising candidate using carefully designed culture models. We will identify the safest and most potent inhibitory compound to use in a novel therapeutic approach that could enhance current treatment options and limit viral spread.

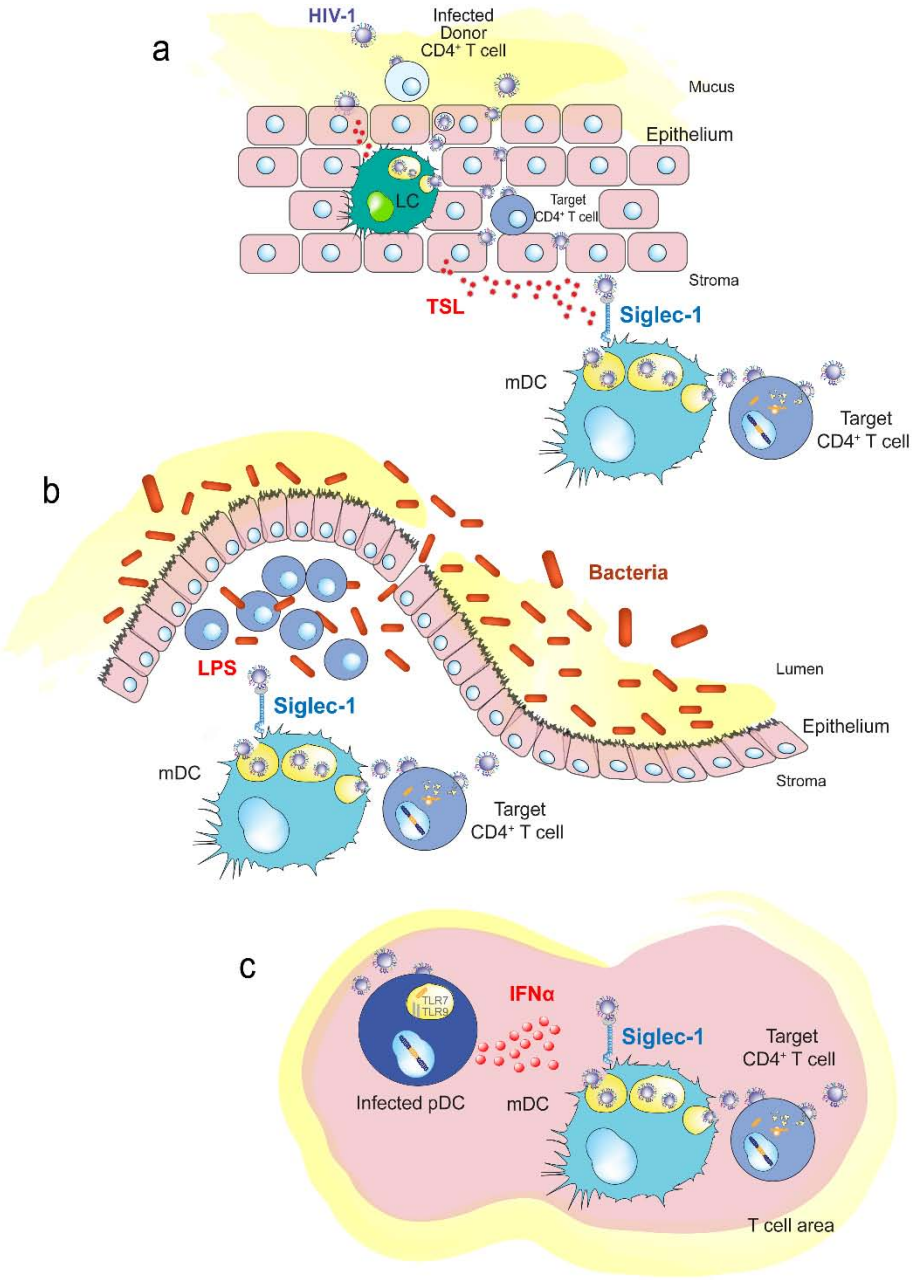


Figure 10. Immune activating signals can induce Siglec-1 expression and contribute to HIV-1 *trans*-infection. A. Human genital mucosal epithelial cells produce TSL in response to HIV-1. This cytokine could induce maturation of Langerhans cells or dermal DCs in the mucosa. **B.** Increased translocation of bacteria from the intestinal lumen after HIV-1 infection augments LPS levels that can stimulate DCs systemically. **C.** HIV-1-exposed plasmacytoid DCs produce IFNα in lymphoid tissues, which trigger activation of bystander DCs and induces Siglec-1 expression. Abbreviations: LC (Langerhans cells), pDC (plasmacytoid DC) TLR (toll-like receptor), TSL (thymic stromal lymphopoietin). Figure from [133].

Chapter 2

HYPOTHESIS & OBJECTIVES

Siglec-1 on DCs recognizes sialyllactose motifs exposed on HIV-1 membrane gangliosides and mediates viral *trans*-infection. Siglec-1 is a cell-surface marker induced on myeloid cells after their exposure to immune-activating signals, such as IFNα, present during HIV-1 acute and chronic infection. However, whether Siglec-1 mediates HIV-1 *trans*-infection in other cells from the myeloid cell lineage, such as monocytes or macrophages, remains unknown.

When active viral replication bursts in HIV-1 infected individuals, peripheral blood monocytes are exposed to circulating immune activating signals present in the plasma, which can efficiently induce Siglec-1 expression on myeloid cells and most likely contribute to viral dissemination. Otherwise, Siglec-1-mediated *trans*-infection could be particularly relevant in lymphoid tissues, the major sites of HIV-1 replication, where myeloid cells migrate and repeatedly establish interactions with $CD4^+$ T cells, the primary targets of HIV-1 productive infection. Importantly, IFNα also accumulates in lymphoid tissues of HIV-1 infected individuals that could induce Siglec-1 expression on myeloid cells, and consequently, contribute to viral spread throughout the course of HIV-1 infection. Despite the prominent role that cell-to-cell viral transmission plays in HIV-1 pathogenesis, no current antiviral treatment targets Siglec-1 viral transfer ability. Thus, designing specific inhibitors against Siglec-1 could pose a novel therapeutic strategy to block cell-to-cell viral transmission. Thus, the objectives of this thesis are:

- **Objective 1:** To determine the expression of Siglec-1 on IFNα activated myeloid cells, such as monocytes and macrophages, and compare it to that of DCs in order to assess if immune activation stimuli present during HIV-1 infection can trigger Siglec-1 induction and mediate viral capture and *trans*-infection.

- **Objective 2:** To elucidate if immune activating signals present in plasma from untreated HIV-1-infected patients could induce Siglec-1 expression and contribute to HIV-1 pathogenesis by modulating *trans*-infection.

- **Objective 3:** To assess whether IFNα stimulation could induce Siglec-1 on myeloid cells isolated from lymphoid tissues, and consequently, contribute to viral spread throughout the course of HIV-1 infection.

- **Objective 4:** To develop sialyllactose-like small compounds (in monovalent and multivalent formulations) and specific blocking mAbs against the V-Set domain of Siglec-1 that could effectively block this specific viral capture and cell-to-cell transmission mechanism.

Chapter 3

RESULTS I

Siglec-1 mediates HIV-1 uptake and *trans*-infection by IFNα-treated myeloid cells

INTRODUCTION

APCs of the myeloid cell lineage, such as DCs, monocytes and macrophages, are essential to initiate innate and adaptive immune responses against invading viruses. While activated $CD4^+$ T cells are susceptible to HIV-1 infection, myeloid cells are refractory to it. This restricted viral infection is mainly due to the presence of cellular factors, such as SAMHD1 or APOBEC3G, that deplete the pool of dNTPs available for reverse transcriptase enzyme and hypermutate viral DNA, respectively, and thus, prevent viral replication [41] [91]. However, the uptake of HIV-1 by myeloid cells and subsequent transfer of virus to uninfected $CD4^+$ T cells results in a burst of virus replication in T cells [164]. Thus, myeloid cells might promote viral pathogenesis through this viral transfer process known as *trans*-infection, which is more efficient than cell-free virus infection of T cells [3][72][103]. This efficient viral dissemination process involves HIV-1 capture and uptake, internalization of intact infectious virus in a compartment and viral release at a cell-to-cell infectious synapse, facilitating new $CD4^+$ T cell infections [164].

We and others have previously identified that Siglec-1 on DCs recognizes syalillactose motifs exposed on HIV-1 membrane gangliosides and mediates viral *trans*-infection [132][135][136]. Siglec-1 is a cell-surface marker induced on myeloid cells after their exposure to immune-activating signals, such as LPS or IFNα [132][135][136], present during HIV-1 acute and chronic infection [150]. IFNα is an antiviral cytokine mainly released by pDCs, which produces up to 1000-fold more IFNα than other cell types upon HIV-1 infection [165][166][167]. Although IFNα inhibits viral replication *in vitro*, it is also a marker of poor prognosis in HIV-1 chronic infected patients [154][167]. Both LPS and IFNα have been shown to induce Siglec-1 expression on DCs enhancing viral capture and *trans*-infection to $CD4^+$ T cells [132][135]. However, whether Siglec-1 mediates HIV-1 *trans*-infection in other cells from the myeloid cell lineage, such as monocytes or macrophages, remains unknown.

The first goal of the present study is to determine the expression of Siglec-1 on IFNα activated myeloid cells such as monocytes and macrophages and compare it to that of

DCs to assess if immune activation stimuli present during HIV-1 infection can trigger Siglec-1 induction and mediate viral capture and *trans*-infection.

MATERIALS AND METHODS

1. Primary cells

All primary cells were cultured in Rosswell Park Memorial Institute (RPMI)-1640 medium with 10% of heat-inactivated Fetal Bovine Serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin (all from Invitrogen). Cells were maintained at 37°C in 5% of CO_2 in a humidified incubator.

1.1. Peripheral Blood Mononuclear Cells (PBMCs)

Buffy coats from HIV-1-seronegative donors were purchased from Banc de Sang i Teixits (BST, Barcelona). PBMCs were obtained by Ficoll-Hypaque (Lymphoprep) density gradient centrifugation of heparin-treated venous blood. $CD3^+$ T cells were depleted from whole blood with RosetteSep Human CD3 Depletion Cocktail (StemCell Technologies) prior to Ficoll-Hypaque density gradient centrifugation in order to increase the proportion of monocytes within the collected PBMCs.

1.2. Monocytes

PBMCs obtained as described above were frozen in 10% dimethyl sulfoxide (DMSO) in heat-inactivated FBS and stored in liquid nitrogen until needed. When DC and macrophage differentiation from fresh isolated monocytes reached day 5, cryopreserved PBMCs from the same donor were thawed by progressively eliminating the DMSO. To limit cell clumping, cells were treated with 50 U/ml benzonase (Novagen) for 5 min at 37°C. Monocytes were positively isolated from thawed PBMCs using $CD14^+$ magnetic beads (Miltenyi Biotec) following manufacturer's protocol, yielding high purity populations (> 95%) based on forward/side scatter flow cytometer analysis. Monocytes were cultured in non-adherent flasks (Nunc, ThermoFisher Scientific) for two days with or without 1000 U/ml of Interferon-2α (IFNα; Sigma-Aldrich) (**Figure 11**).

1.3. Monocyte-derived dendritic cells (DCs)

Monocytes were positively isolated from fresh PBMCs using $CD14^+$ magnetic beads (Miltenyi Biotec) as previously described, cultured at 0.8 x 10^6 cells/ml and differentiated into DCs adding 1000 U/ml of GM-CSF plus 1000 U/ml of Interleukin-4 (IL-4; both from R&D Systems) (**Figure 11**). Cells were cultured for 7 days in non-adherent flasks, and media and cytokines were renewed every two days. At day five, DCs were stimulated adding to the culture medium 1000 U/ml of IFNα or left untreated. After 48 h of stimulation, cells were designated as LPS-mature DCs (LPS DCs), IFNα-treated DCs (IFNα DCs) or immature DCs (iDCs), respectively.

1.4. Monocyte-derived Macrophages (MØ)

Monocytes were cultured at 0.8 x 10^6 cells/ml and differentiated into macrophages adding 100 ng/ml of MCS-F (Preprotech) in adherent T75 flasks (Nunc, Thermofisher Scientific) disposed horizontally to allow monocyte attachment. Cells were cultured for 7 days, and cytokines and media were renewed every two days. At day five, macrophages were stimulated with 1000 U/ml of IFNα for two days (**Figure 11**).

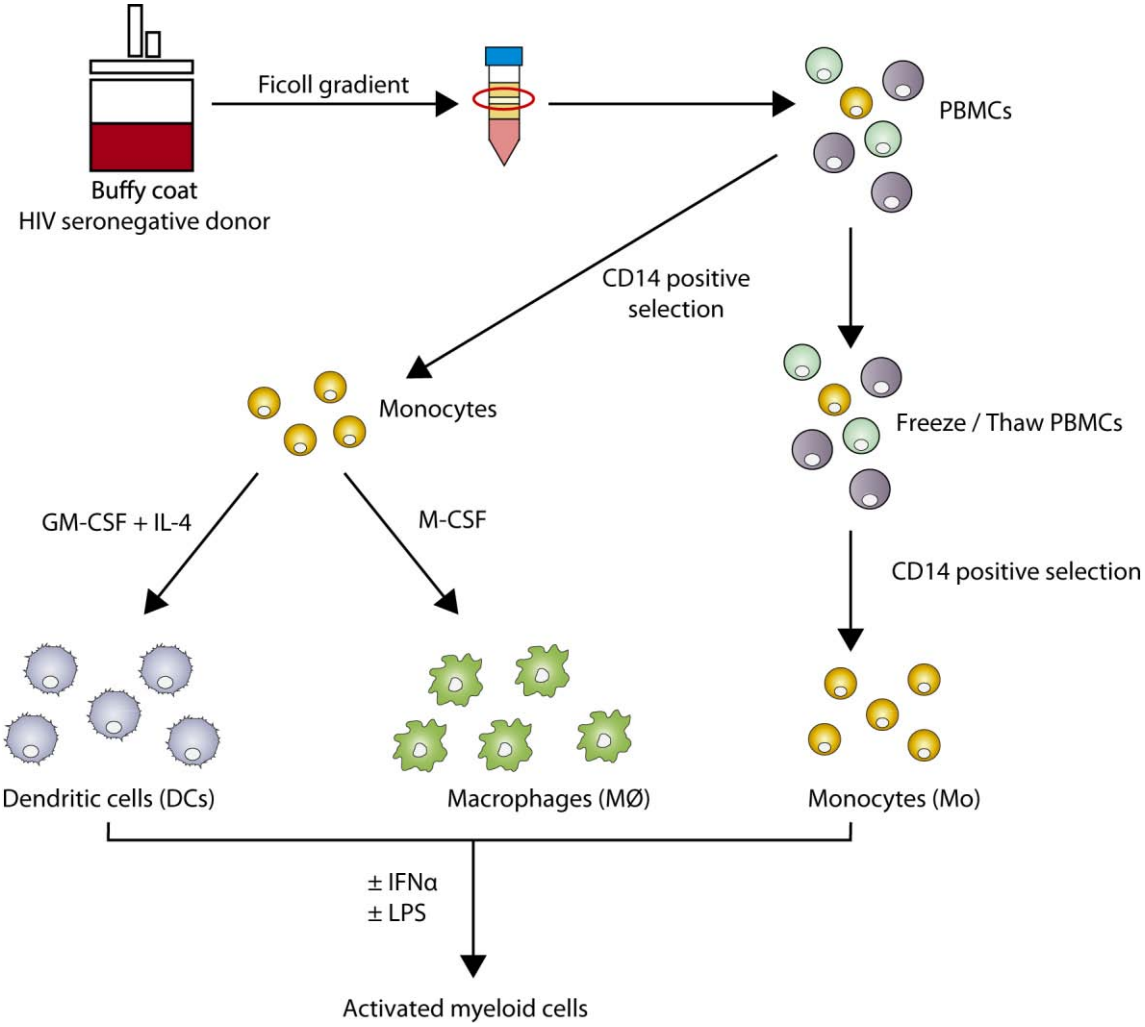


Figure 11. Primary cell culture scheme. Monocyte isolation procedure from PBMCS of HIV-1 seronegative buffy coats, and cell culture method for monocytes, DCs and macrophages.

1.5. Plasmacytoid Dendritic Cells (pDCs)

pDCs were negatively isolated from fresh PBMCs obtained as described above using Plasmacytoid Dendritic Cell Isolation magnetic beads (Miltenyi Biotec), following manufacturer's protocol. Experiments with pDCs were performed the same day of their isolation.

1.6. $CD4^+$ T lymphocytes

$CD4^+$ T lymphocytes were obtained by depleting $CD8^+$ T cells from whole blood with RosetteSep Human CD8 Depletion Cocktail (StemCell Technologies) prior to Ficoll Hypaque density gradient centrifugation. $CD8^+$ T depleted PBMCs were cultured at 1.25 x 10^6 cells/ml in RPMI-1640, containing 10% FBS, 100 U/ml of penicillin, 100 µg/ml of

streptomycin and 10 U/ml IL-2 (Roche) achieving a 95% of $CD4^+$ T cell purity. Three days before the experiment, 3 µg/ml of phytohemagglutinin (PHA; Sigma-Aldrich) was added to the medium.

2. Cell lines

2.1. Adherent cell lines

HEK-293T and TZM-bl (obtained through the US National Institutes of Health [NIH] AIDS Research and Reference Reagent Program) were maintained in Dulbecco's Modified Eagle Medium (D-MEM; Invitrogen) containing 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin.

HEK-293T cell line originated from Human Embryonic Kidney was used to generate viral stocks by transfection of viral plasmid DNA [168–171]. HEK-293T cells were detached using calcium chelating agent Versene 1x (Invitrogen) for 3 min at 37°C and split twice a week.

TZM-bl cell line is a HeLa cell line generated by introducing separate integrated copies of the luciferase and ß-galactosidase genes under the control of the HIV-1 promoter [172–174]. Thus, HIV-1 infection in these cells can be easily monitored by luminescence or colorimetric assays. This cell line was used to determine the infectivity of viral stocks and to perform *trans*-infection assays. TZM-bl cells were split twice a week using the protease trypsin 1x (Invitrogen) for 10 min at 37°C.

2.2. Suspension cell lines

Lymphoblastic cell lines MOLT-4 (obtained through ATCC) and chronically HIV-1_{Bal}-infected MOLT-4 (kindly provided by Dr. J. Blanco) were maintained in RPMI-1640 medium containing 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin. To generate the chronically HIV-1_{Bal}-infected MOLT-4 cell line, MOLT-4/CCR5 T cells were infected with a recombinant virus carrying the envelope sequence of the R5 HIV-1 strain BaL in a HIV-1_{HXB2} backbone. This HIV-1_{Bal}-producing cell line was used to infect pDCs.

In all cell lines indicated above, cells were diluted 1:10 in fresh media twice a week to maintain cell culture in optimal conditions.

3. Immunophenotype

Cell staining was performed prior blocking Fc receptors with 1 mg/ml of human Ig Gs (h Ig Gs; Privigen, Behring CSL) for 20 min at Room Temperature (RT). Subsequently, 2 x 10^5 cells were stained at 4°C for 30 min with the monoclonal antibodies (mAbs) indicated in **Table 2.** For intracellular staining, cells were fixed and permeabilized (Fix & Perm; Invitrogen) prior mAb incubation, following manufacturer's protocol.

Then, cells were extensively washed and fixed with 1 % formaldehyde in Phosphate Buffered Saline (PBS). Matched Isotype Ab controls were used. Cells were analyzed with FACSCalibur using CellQuest (Becton Dickinson) and FlowJo software to evaluate collected data.

Table 2. Panel of monoclonal antibodies used to immunophenotype cells.

Monoclonal Antibody (mAb)	Fluorochrome	Clone	Brand
Siglec-1	PE	7-239	AbDSerotec
P24	PE	KC57	Beckman Coulter
CD2	PerCPCy5.5	RPA-2.10	Becton Dickinson

All mAbs were titrated to determine the optimal antibody dilution, which give the brightest staining with the minimum background. Optimal dilution was calculated choosing the one with the highest ratio signal to noise.

3.1. Siglec-1 surface expression analysis by flow cytometry

Fc receptors from 2 x 10^5 cells were blocked as previously described. After washing, cells were incubated with mAb 7-239 α-Siglec-1-PE or matched isotype-PE control (AbD Serotec) for 30 min at 4°C. Cells were fixed with 1% formaldehyde and analyzed by flow cytometry (FACS Calibur, Beckton Dickinson).

The mean number of Siglec-1 mAb binding sites per cell (antibody-to-PE ratio 1:1) was obtained with a Quantibrite kit (Becton Dickinson) [175]. First, a standard linear regression was built using four different beads conjugated with known numbers of PE molecules per bead. We could then extrapolate the geometrical mean fluorescence obtained to known number of PE molecules per bead. Using the same settings and voltage, cell samples were collected. Regression of the geometrical mean fluorescence

intensity obtained for cells to the standard linear regression curve built allowed us to quantify the number of Siglec-1 mAb binding sites per cell of interest. Geometrical mean fluorescence intensity of an isotype control was subtracted for each sample. Collected data was analyzed using CellQuest software.

Before assessing Siglec-1 induction in myeloid cells, different isolation techniques were tested to see if Siglec-1 up-regulation could take place due to technical procedures.

We assessed magnetic bead isolation because these procedures yield highly pure populations (> 95%). Siglec-1 expression was assessed by flow cytometry before and after monocyte isolation by either negative or positive selection procedures. Of note, neither negative nor positive isolation with magnetic beads up-regulated basal Siglec-1 expression (**Figure 12**).

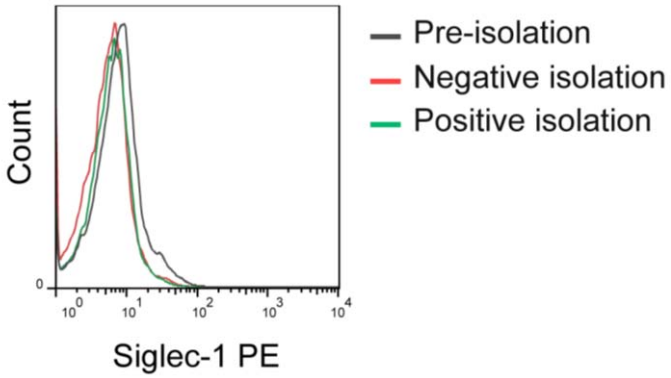


Figure 12. Siglec-1 expression on monocytes before and after monocyte isolation with distinct procedures. Representative profiles of Siglec-1 staining in monocytes before (grey) and after positive (green) or negative (red) isolation with magnetic beads, assessed by flow cytometry.

In order to obtain enough and highly pure monocyte populations we decided to isolate monocytes from buffy coats by positive selection with magnetic beads as described previously.

4. Plasmids and viral stocks

HIV-1_{NL4-3}, and GFP-viral like particles ($VLP_{HIV-Gag-eGFP}$) stocks were produced by transfecting HEK-293T cell line with the molecular clones pNL4-3 and pGag-eGFP, respectively. Both plasmids were obtained from the NIH AIDS Research and Reference

Reagent Program. HIV-$1_{NL4-3-Cherry}$ stock was produced by HEK-293T cotransfection of $pCHIV_{mCherry}$ and pCHIV plasmids, kindly provided by Dr. B. Müller, in a ratio 1:1 [176]. HEK-293 T cells were transfected with calcium phosphate (Calphos Mammalian kit, Clontech) in T75 flasks using a total of 30 µg of plasmid DNA.

After 48 h, supernatants containing virus or VLPs were filtered (Millex HV, 0.45 µm) and viral stocks were stored in 2 ml cryopreserved tubes at – 80 °C until use. Successful transfection in the case of $VLP_{HIV-Gag-eGFP}$ and HIV-$1_{NL4-3-Cherry}$ could be followed by fluorescence microscopy.

The $p24^{Gag}$ content of the viral and VLPs stocks was quantified by a $p24^{Gag}$ Enzime-Linked Immunosorbent assay ($p24^{Gag}$ ELISA; Perkin-Elmer). Because VLPs contain the unprocessed Gag polyprotein and the anti-p24 ELISA antibodies do not recognize that polyprotein as efficiently as the processed $p24^{Gag}$, there is an underestimation in this particular viral stock. However, $p24^{Gag}$ quantification among different VLPs viral stocks allowed us to add equivalent amounts of VLPs in each experiment.

To obtain the titer of the infectious viral stocks, a TZM-bl infectivity assay was performed (modified from [177]). The TZM-bl reporter cell line enables to quantify the infectivity of viral stocks by luciferase activity. Serial two-fold dilutions of each viral stock were made in duplicate in 96-well optical bottom culture plates in a total volume of 100 µl of growth medium for a total of 11 dilutions steps. Freshly trypsinized cells (10,000 TZM-bl in 100 µl of media) were added to each well. After a 48-h incubation, 100 µl of Bright Glo reagent (BrightGlo luciferase system, Promega) was added to the cells. After a 2-min incubation at RT to allow cell lysis, luciferase activity was measured with a Fluoroskan Ascent FL luminometer (Thermo Labsystems). Background values consisting of non-pulsed TZM-bl were determined and subtracted from positive values. By revealing luciferase activity, we calculated $TCID_{50}$/ml and extrapolated the Multiplicity of Infection (MOI) of a reference virus. All viral stocks used in the assays were normalized to the reference virus.

5. Siglec-1 induction by supernatants from HIV-1-infected pDCs

A total of 0.1 x 10^6 pDCs were co-cultured with 0.1 x 10^6 HIV-1_{Bal}-infected MOLT-4 (ratio 1:1) for 24 h at 37°C. As a negative control, 0.1 x 10^6 pDCs were co-cultured with

0.1 x 10^6 uninfected MOLT-4. In parallel, 0.1 x 10^6 pDCs were pre-treated with 10 µg/ml of mAb against CD4 (RPA T-4) to avoid pDC infection or 10 µg/ml of isotype mAb (both from Becton Dickinson) for 10 min at 37°C. After 24 h of pDC-MOLT-4 co-culture, supernatants were collected and transferred to 0.2 x 10^6 immature DCs. Siglec-1 expression of DCs was assessed by flow cytometry after 24 h of incubation with supernatants (**Figure 13**).

6. VLP and HIV-1 binding and uptake assays

Cells were pre-treated at 4°C for 30 min with 10 µg/ml of the functional grade mAb α-Siglec-1 7-239, Ig G1 Isotype control or left untreated. Viral binding assays were performed at 4°C in order to avoid viral internalization, while viral uptake assays were performed at 37°C to allow internalization. To assess HIV-1_{NL4-3} binding and uptake, 4 x 10^5 cells were pulsed with 970 ng of $p24^{Gag}$ for 4 h. After extensive washing, cells were lysed with 0.5% Triton X-100 at 0.5 x 10^6 cells/ml to measure $p24^{Gag}$ antigen by an ELISA. To analyze viral degradation, cells were pre-incubated with 250 nM of bafilomycin A1 (Sigma-Aldrich) during 30 min at 37°C and then exposed to HIV-1_{NL4-3} in the presence of the drug or left untreated. To determine VLP binding and uptake, 2 x 10^5 cells were pulsed with 10 ng of VLPs for 3 h. After viral pulse, cells were extensively washed with PBS and analyzed by Flow Activating Cytometer Sorting (FACS) (**Figure 14**). All experiments were performed in FACS tubes in order to assure completely removal of unbound virus. Previous experiments performed in Eppendorf tubes, which only allow washing cells with up to 1 ml of PBS, demonstrated inefficient viral washing compared to FACS tubes, which allow up to 3 ml of PBS.

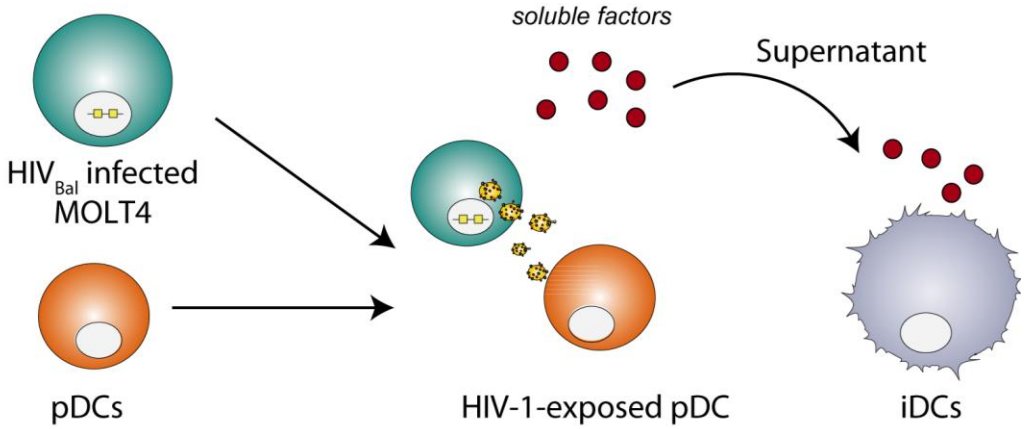


Figure 13. Schematic representation of the experimental procedure used in Siglec-1 induction by supernatants from HIV-1-exposed pDCs. HIV-1$_{Bal}$-infected MOLT-4 were co-cultured with fresh isolated pDCs and supernatants released were collected. Supernatants were transferred to immature DCs.

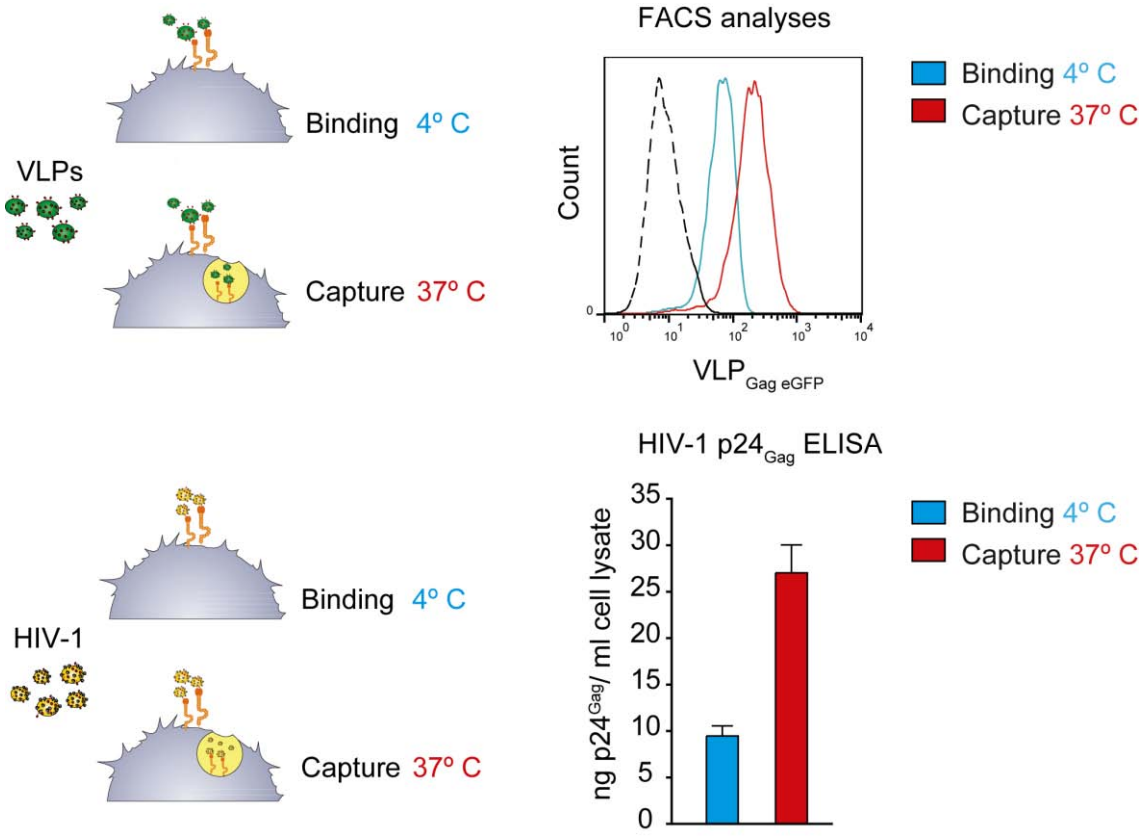


Figure 14. Schematic representation of the experimental procedure used in viral binding and capture assays. Cells were pulsed with VLPs or HIV-1$_{NL4-3}$ at 4°C to perform binding assays or at 37°C to perform capture assays. VLP and HIV-1$_{NL4-3}$ binding and capture assays were revealed by FACS and p24^{Gag} ELISA, respectively.

7. HIV-1 *trans*-infection assays

To evaluate HIV-1 transmission, 0.4 x 10^6 cells were pre-treated with 10 µg/ml of the mAbs α-Siglec-1 7-239, Ig G1 Isotype control or left untreated for 30 min at 4°C. Cells were pulsed with 970 ng of HIV-1_{NL4-3} for 4 h and extensively washed. Then, cells were co-cultured at a ratio 1:1 or 1:5 with a reporter cell line TZM-bl, which enables quantification of viral infection by luminescence. Cells were assayed for luciferase activity 48 h later (**Figure 15**). Background values consisting of non-HIV-1 pulsed co-cultures were measured for each experiment. Of note, we chose the CXCR4-tropic virus NL4-3 and short period co-culture assays to avoid productive *cis*-infection of myeloid cells and focus on *trans*-infection processes.

To assess the antiviral impact of IFNα on viral transmission, DC – activated $CD4^+$ T-cell co-cultures (ratio 1:1) were set up as described above, but maintaining 10 U/ml IFNα in the culture. Moreover, to determine viral transmission capacity between $CD4^+$ T cells and cell free virus infection, $CD4^+$ T lymphocytes were also pulsed with the same amount of infectious virus and co-cultured with $CD4^+$ T lymphocytes (ratio 1:1) or left alone, respectively. After 2 days of co-culture, cells were fixed, permeabilized and stained with mAb α-CD2 PerCP-Cy5.5 (clone RPA-2.10; Becton Dickinson) and mAb α-p24 PE (clone KC57-RD1; Beckman Coulter) to distinguish containing virus $CD4^+$ T cells. Cell staining was revealed by FACS on a Becton Dickinson LSRII.

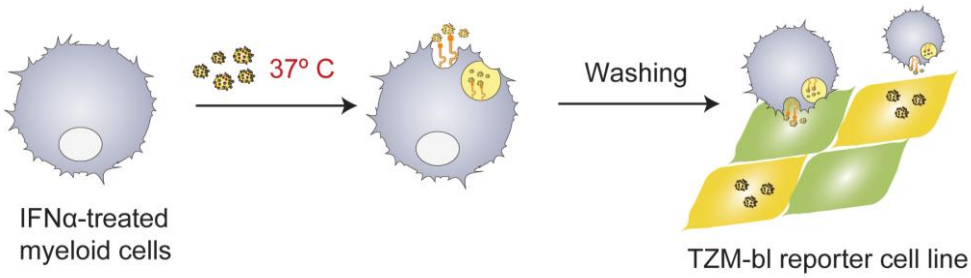


Figure 15. Schematic representation of the experimental procedure used in viral *trans*-infection. IFNα-activated myeloid cells were pulsed with HIV-1_{NL4-3} at 37°C for 4 h. After extensive washing, cells were co-cultured with TZM-bl reporter cell line for 48 h.

8. Confocal Immunofluorescence Microscopy

4 x 10^5 cells were incubated with HIV-$1_{NL4-3-Cherry}$ for 4 h at 37°C. After extensive washing, cells were fixed and permeabilized and stained with mAbs α-HLA-DR-Alexa

647 (Clone L243, Biolegend) to reveal cell membranes and α-Siglec-1 7-239 Alexa 488 (AbD Serotec). Cells were fixed in 1% formaldehyde, cytospun into coverslips (Thermo Scientific) and mounted in mounting media containing fluorescent DAPI (Invitrogen) in order to stain cell nuclei. Cells stained were analyzed with an Ultraview ERS Spinning Disk System (Perkin-Elmer) mounted on a Zeiss Axiovert 200M inverted microscope at the Advanced Fluorescence Microscopy Unit (AFMU) of the Molecular Biology Institute of Barcelona (IBMB). To obtain 3D reconstructions, confocal z-stacks were acquired at 0.3 µm steps using a 63x objective. Microscopy images were analyzed with Volocity software (Perkin-Elmer) employing maximum fluorescent intensity projection.

9. Statistical analysis

We analyzed mean changes using a paired *t*-test, which was considered significant at $P< 0.05$. Significant mean changes from 100% of the data normalized to percentages were assessed with a one sample *t*-test, considered significant at $P< 0.04$. All analyses and figures were generated with the GraphPad Prism v5.0b Software.

RESULTS

1. Siglec-1 is up-regulated on dendritic cells even at low doses of IFNα

To examine whether physiological levels of IFNα (range from 10 U/ml to 100 U/ml) [9] could up-regulate Siglec-1 expression on DCs, immature DCs were activated with decreasing amount of IFNα. When we analyzed Siglec-1 expression, IFNα-stimulated cells presented high Siglec-1 expression levels even at low concentrations (**Figure 16**).

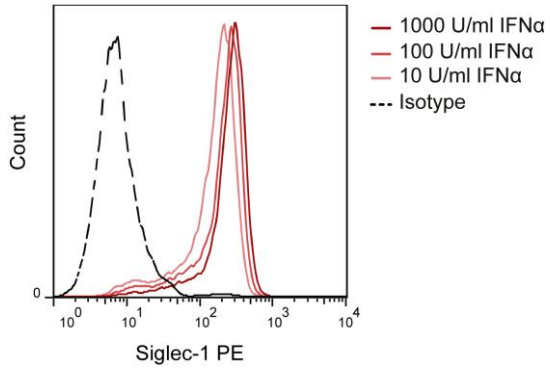


Figure 16. Caption overleaf.

Figure 16. (From previous page). Siglec-1 expression upon DC exposure to different concentrations of IFNα. Cell surface expression of Siglec-1 on DCs exposed to different IFNα concentrations analyzed by FACS with mAb 7–239-PE. Representative profile of Siglec-1 staining on IFNα-activated DCs derived from one donor.

To assess if this induction could take place in a more physiological setting, we isolated pDCs, which secrete type I IFN upon HIV-1 infection [178]. pDCs were co-cultured with a chronically HIV-1-infected T-cell line or with uninfected cells for 24 h. Thereafter, supernatants from these co-cultures were collected and transferred to immature DCs to assess Siglec-1 expression by FACS 24 h later. Siglec-1 was increased on DCs incubated with supernatants from HIV-1-exposed pDCs compared with those supernatants from unexposed pDCs or pDCs co-cultured with uninfected cells (**Figure 17**). Blockage of pDC viral infection with a mAb against CD4 reduced Siglec-1 induction on DCs, while isotype control treatment had no effect (**Figure 17**). Thus, HIV-1 exposition of pDCs results in the secretion of soluble factors, presumably type I interferons that clearly induce Siglec-1 expression on DCs.

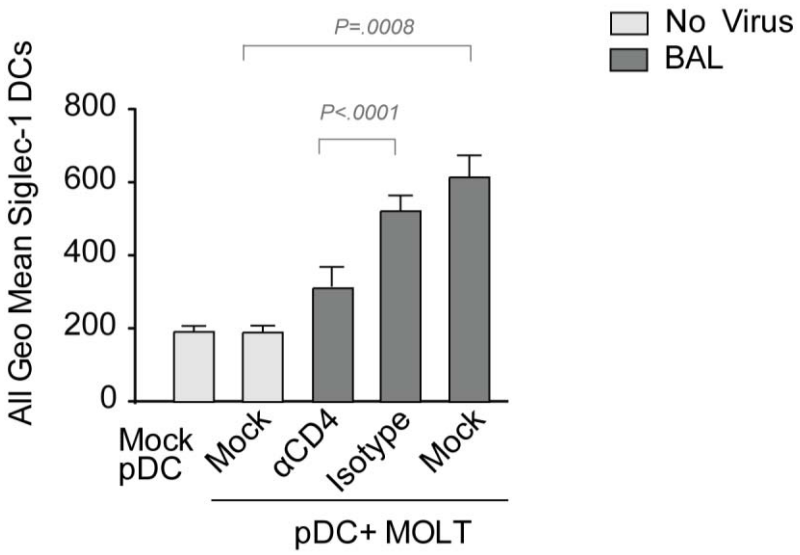


Figure 17. Induction of Siglec-1 by supernatants of HIV-1_{BAL}-exposed-pDCs. Siglec-1 cell surface expression of DCs analyzed by FACS with mAb 7–239-PE. All Geometric mean fluorescence intensity (MFI) of Siglec-1. Data show mean values and SEM from two experiments including cells from 2 pDC donors against 6 monocyte-derived DCs donors.

2. Siglec-1 is up-regulated on myeloid cells upon IFNα treatment

When we compared Siglec-1 expression levels on distinct myeloid cells activated in the presence of IFNα, we observed a 17-fold up-regulation in DCs and a twofold up-

regulation in macrophages and monocytes (**Figure 18A**). We have previously reported that Siglec-1 expression levels determine the capacity of DCs to capture HIV-1 [9]. To test whether this also holds true for monocytes and macrophages, we first compared the density of Siglec-1 surface expression applying a quantitative FACS assay that determines the absolute number of Siglec-1 antibody binding sites. This number was highest in IFNα-activated monocyte-derived DCs, followed by macrophages and monocytes (**Figure 18B**).

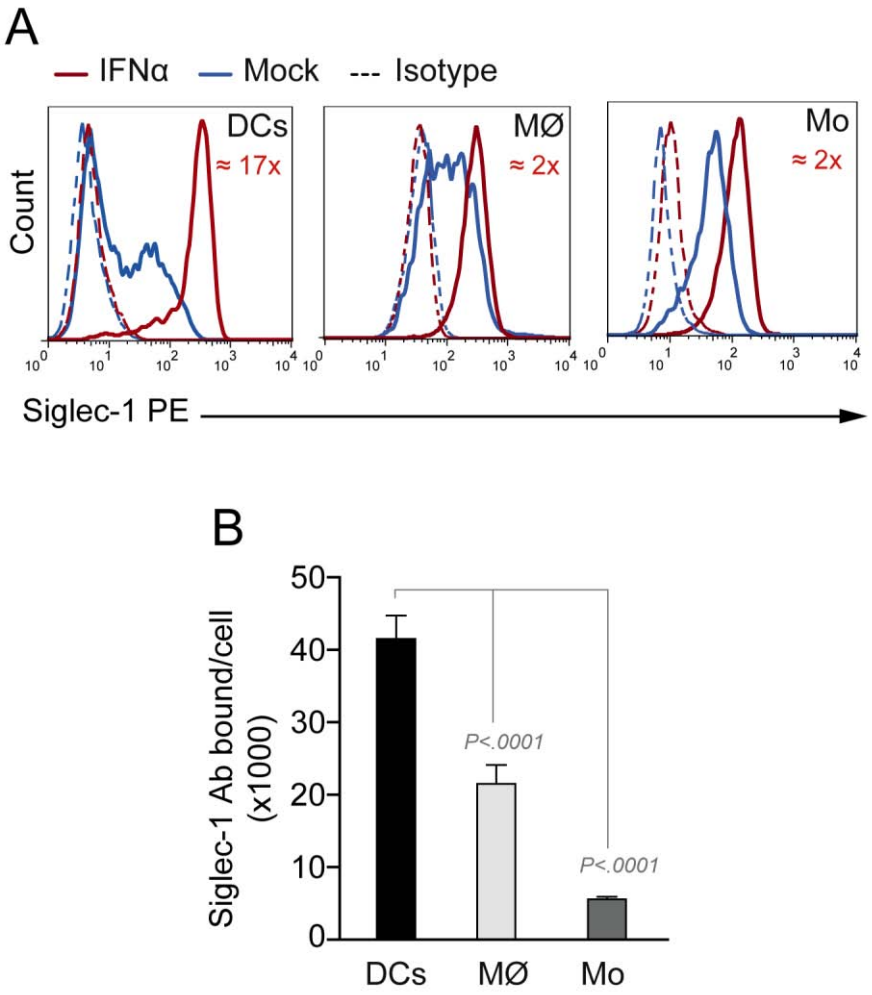


Figure 18. Siglec-1 expression on different myeloid cells (DCs, macrophages, monocytes) upon IFNα stimulation. A. Representative profile of Siglec-1 staining in distinct myeloid cells cultured with or without 1000 U/ml of IFNα and assessed by FACS. Staining of matched-isotype control is also shown. The mean fold increase in fluorescence after IFNα treatment of cells derived from three donors is shown in red numbers. **B.** Mean number of Siglec-1 antibody binding sites per cell displayed by different myeloid cells exposed to 1000 U/ml of IFNα for 48 h and assessed by quantitative FACS analysis. Data show mean values and SEM from four experiments including cells from 12 donors. Statistical differences were assessed with a paired *t* test.

3. Siglec-1 mediates HIV-1 capture by IFNα-treated myeloid cells

Next, we analyzed binding of infectious HIV-1 to IFNα-activated myeloid cells. HIV-1 was incubated with the cells for 4 h at 4°C to avoid viral internalization and cell-associated $p24^{Gag}$ was quantified by ELISA after extensive washing. Consistent with their respective Siglec-1 expression levels (**Figure 18B**), IFNα-activated monocyte-derived DCs showed a higher HIV-1 binding capacity than monocytes (**Figure 19A**). To investigate whether this binding was specific for Siglec-1, cells were pre-treated with a mAb against Siglec-1. This treatment led to a reduction of HIV-1 binding by 83% in all cases, while isotype control treatment had no inhibitory effect (**Figure 19B**).

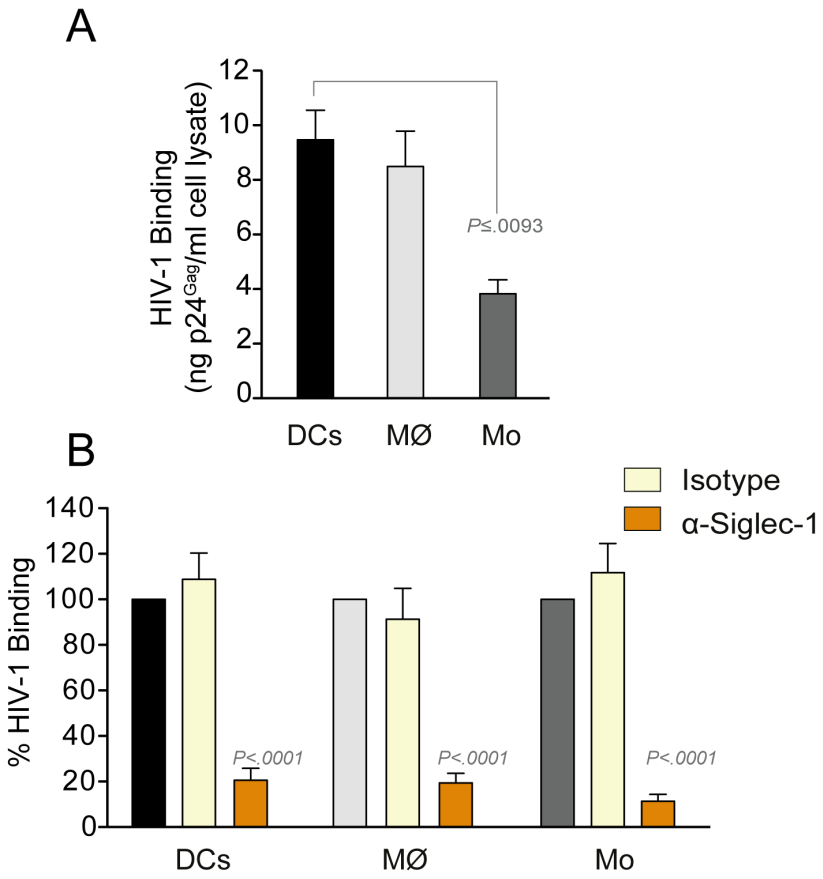


Figure 19. Siglec-1 mediates HIV-1 binding by IFNα-treated myeloid cells. A. Comparative binding of HIV-1_{NL4-3} to different myeloid cells previously exposed to 1000 U/ml of IFNα for 48 h. Cells were cultured with HIV-1_{NL4-3} for 4 h at 4°C, washed and lysed to measure $p24^{Gag}$ by ELISA. Data show mean values and SEM from two experiments including cells from six donors. **B.** Relative binding of HIV-1_{NL4-3} by different IFNα-treated myeloid cells that had been pre-incubated with 10 µg/ml of the indicated mAbs before HIV-1 exposure for 4 h at 4°C as described in **A.** To compare the effect of the mAbs in different myeloid cells, values were normalized to the level of HIV-1 binding by mock-treated cells (set to 100%). Data show mean values and SEM from two experiments including cells from six donors. Statistical differences were assessed with a paired *t* test in **A**, and with a one sample *t*-test in **B**.

Similar results were obtained with fluorescent VLPs, which lack the viral envelope glycoprotein but carry sialyllactose-containing gangliosides recognized by Siglec-1 (**Figure 20**). These data indicate that Siglec-1 is the main molecule responsible for HIV-1 capture by IFNα-activated myeloid cells, and that its expression correlates with the viral binding capacity of the respective cell type.

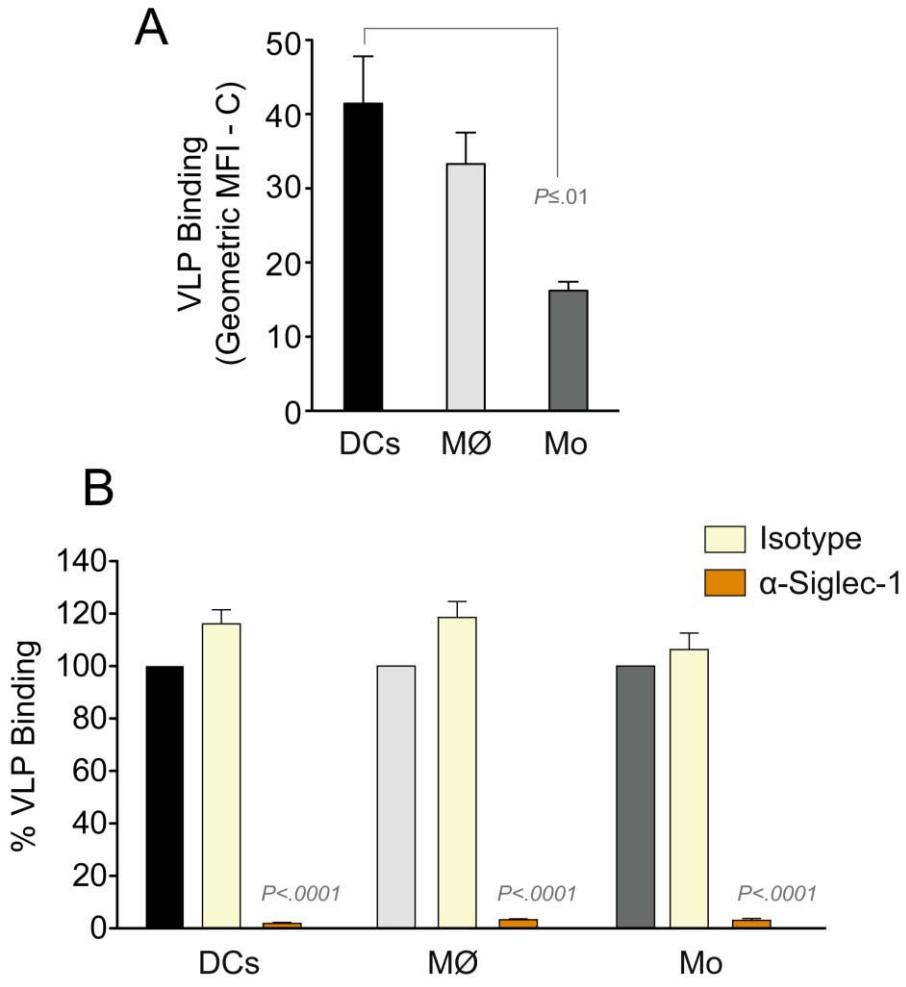


Figure 20. Siglec-1 mediates VLP binding by IFNα-treated myeloid cells. A. Comparative binding of fluorescent VLPs to different myeloid cells previously exposed to 1000 U/ml of IFNα for 48 h. Cells were pulsed with VLPs for 3 h at 4°C and then assessed by FACS to measure the geometric mean fluorescence intensity (MFI). Data show mean values and SEM from two experiments including cells from six donors. **B.** Relative binding of VLPs by different IFNα-treated myeloid cells that had been pre-incubated with 10 µg/ml of the indicated mAbs before VLP exposure for 3 h at 4°C. Values are normalized to the level of VLP captured by mock-treated cells (set at 100%). Data show mean values and SEM from two experiments including cells from six donors. Statistical differences were assessed with a paired *t* test in **A**, and with a one sample *t*-test in **B**.

4. Siglec-1 mediates viral uptake into a storage compartment and enhances HIV-1 *trans*-infection especially in IFNα-treated monocytes and DCs

Having established Siglec-1-dependent virus binding in all three types of myeloid cells, we performed uptake experiments at 37°C to follow the fate of the bound virus. IFNα-activated myeloid cells were incubated with HIV-1 for 4 h at 37°C and cell-associated $p24^{Gag}$ was quantified by ELISA after extensive washing (**Figure 21A**). Monocyte-derived DCs and monocytes contained similar amounts of HIV-1, while macrophages displayed lower uptake (**Figure 21A**). Treatment with Bafilomycin A1, an inhibitor of lysosomic degradation, only increased the level of cell-associated virus in macrophages (**Figure 21B**). Thus, faster viral degradation in macrophages accounts for the reduced cell-associated virus observed in this cell type (**Figure 21A**). HIV-1 uptake was strongly inhibited by a mAb against Siglec-1 in all cases (**Figure 21C**).

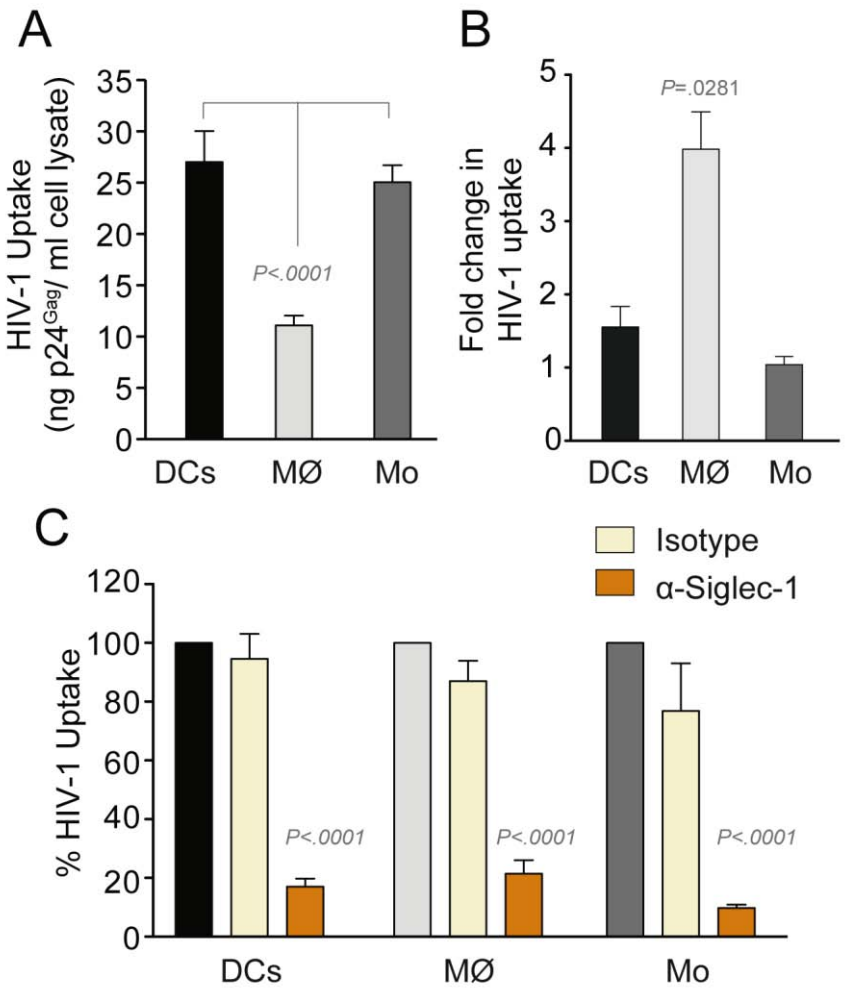


Figure 21. Siglec-1 mediates HIV-1 uptake in IFNα-treated monocytes and DCs. A. Uptake of HIV-1_{NL4-3} by different myeloid cells exposed to IFNα. Cells were cultured with HIV-1 to measure $p24^{Gag}$ by ELISA. Mean values and SEM from four experiments include cells from 12 donors. **B.** Fold change in HIV-1_{NL4-3} uptake of cells treated with bafilomycin A1 compared to untreated cells. Mean values and SEM include cells from three donors. **C.** Relative uptake of HIV-1_{NL4-3} by IFNα-treated myeloid cells pre-incubated with the indicated mAbs. Values are normalized to the level of HIV-1 uptake by mock-treated cells (set at 100%). Mean values and SEM from two experiments include cells from six donors. Statistical differences were assessed with a paired *t* test in **A** and with a one sample *t*-test in **B** and **C**.

Similar results were obtained when we performed uptake experiments with fluorescent VLPs (**Figure 22A**), with macrophages showing residual capture in the presence of the mAb (**Figure 22B**).

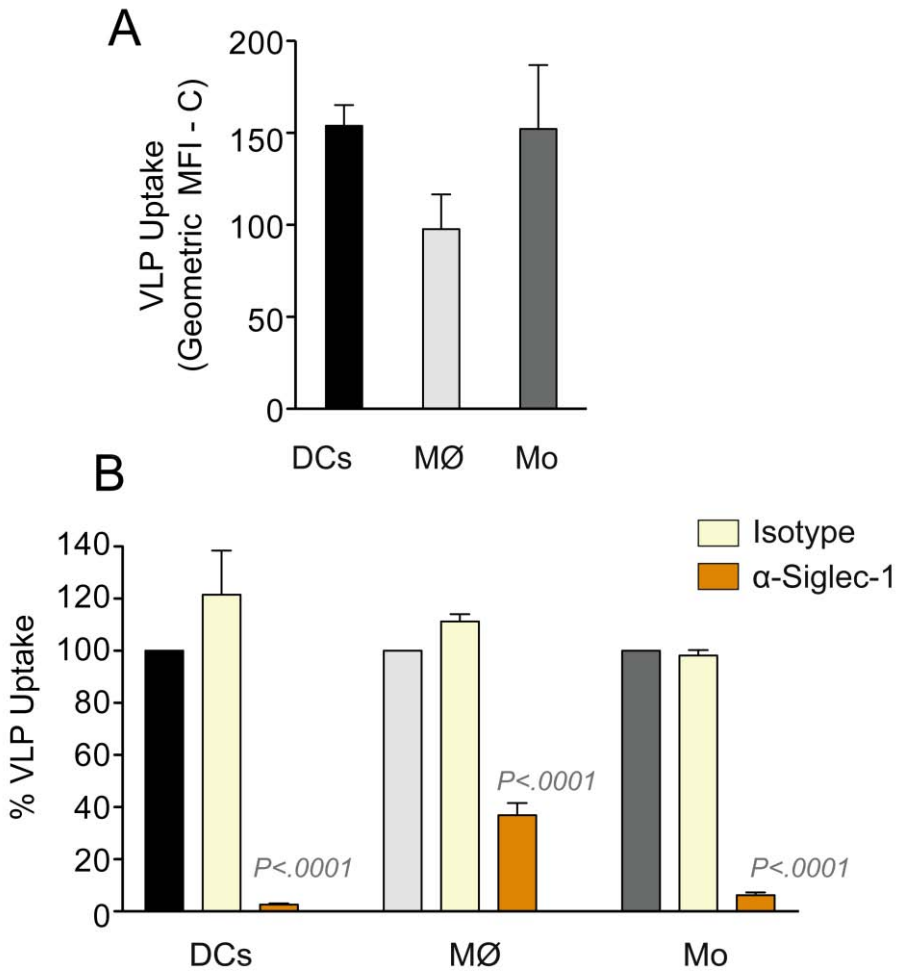


Figure 22. Siglec-1 mediates VLP uptake in IFNα-treated monocytes and DCs. A. Uptake of fluorescent VLPs by different myeloid cells previously exposed to 1000 U/ml of IFNα for 48 h. Cells were pulsed with VLPs for 3 h at 37°C and assessed by FACS to measure the geometric mean fluorescence intensity (MFI). Data show mean values and SEM from two experiments including cells from six donors. **B.** Relative uptake of VLPs by different IFNα- treated myeloid cells that had been pre-incubated with 10 μg/ml of the indicated mAbs before VLP exposure for 3 h at 37°C. Values are normalized to the level of VLP captured by mock-treated cells (set at 100%), and P values are calculated with a one sample t-test. Data show mean values and SEM from two experiments including cells from six donors. Statistical differences were assessed with a paired *t* test in **A** and with a one sample *t*-test in **B**.

To elucidate the HIV-1 trafficking differences in macrophages compared to other myeloid cells, we investigated viral uptake by confocal microscopy. IFNα-treated myeloid cells were pulsed with fluorescent HIV-$1_{NL4\text{-}3\text{-}Cherry}$ for 4 h at 37°C and subsequently stained with mAbs against Siglec-1 and against HLA-DR to reveal cellular membranes (**Figure 23, top images**). While most of the monocyte-derived DCs and monocytes accumulated HIV-$1_{NL4\text{-}3\text{-}Cherry}$ within a sac-like compartment enriched in Siglec-1, macrophages exhibited a more scattered pattern for cell-associated HIV-$1_{NL4\text{-}3\text{-}}$

$_{Cherry}$ (**Figure 23**). Thus, the complementary approaches of confocal microscopy analysis and viral uptake experiments indicate that Siglec-1 is essential for HIV-1 capture in all myeloid cells, while it is not sufficient for further downstream uptake and trafficking involved in the formation of a condensed viral compartment.

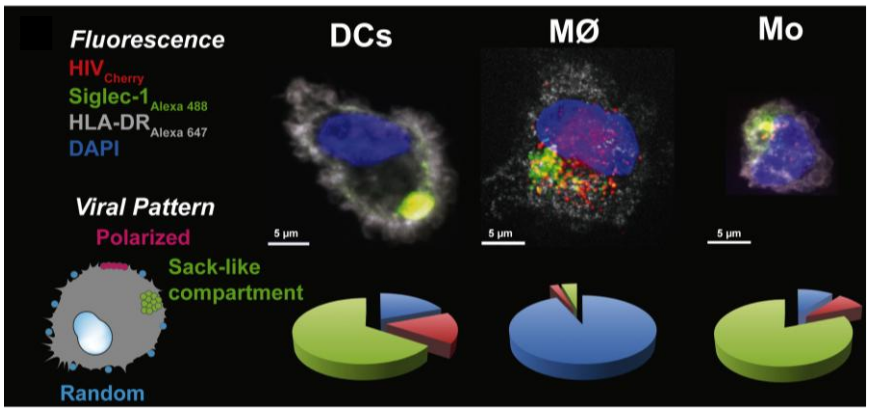


Figure 23. Siglec-1 mediates viral uptake into a storage compartment especially in IFNα-treated monocytes and DCs. Confocal microscopy analysis of different IFNα-treated myeloid cells pulsed with HIV-$1_{NL4\text{-}3\text{-}Cherry}$ and stained for Siglec-1 (Alexa 488), HLA-DR (Alexa 647) and DAPI. (Top) Representative viral pattern for each kind of myeloid cell analyzed, showing maximum fluorescence intensity of four channels. (Bottom) Percentage of myeloid cells with distinct viral patterns: random distribution, polarized accumulation, and sac-like compartment formation, as illustrated in the left drawing. Mean values of 50 cells from two different donors are shown.

We next assessed the capacity for HIV-1 *trans*-infection by the different myeloid cell types. Cells were pulsed with equal amounts of the X4-tropic virus NL4-3 followed by extensive washing and were subsequently co-cultured with a $CD4^+$ reporter cell line for two days. Monocyte-derived DCs had the highest capacity for *trans*-infection followed by monocytes, while macrophages showed only weak *trans*-infection capacity (**Figure 24A**), consistent with their faster viral degradation kinetics (**Figure 21B**). However, *trans*-infection depended on Siglec-1 in all cases (**Figure 24B**).

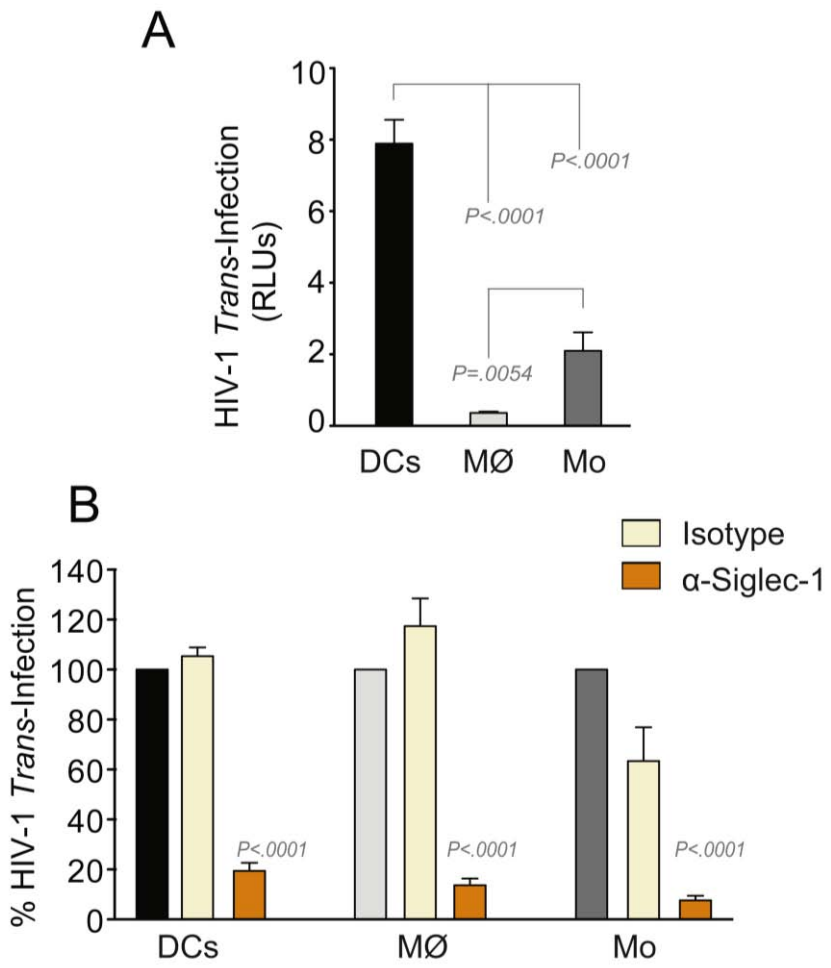


Figure 24. Siglec-1 enhances HIV-1 *trans*-infection especially in IFNα-treated monocytes and DCs. A. HIV-1 transmission from IFNα-treated myeloid cells to a luciferase reporter $CD4^+$ T cell line. HIV-1 infection was determined by induced luciferase activity in relative light units (RLUs). Mean values and SEM from four experiments include cells from 12 donors. **B.** Relative HIV-1 transmission from IFNα-treated myeloid cells pre-incubated with the indicated mAbs. Values are normalized to the level of HIV-1 *trans*-infected by mock-treated cells. Mean values and SEM from two experiments include cells from six donors. Statistical differences were assessed with a paired *t* test in **A** and with a one sample *t*-test in **B**.

5. IFNα antiviral activity does not inhibit Siglec-1 mediated HIV-1 transmission to $CD4^+$ T cells

Despite the enhanced *trans*-infection observed for IFNα-treated myeloid cells expressing Siglec-1, the presence of this antiviral cytokine during *trans*-infection could block viral transmission. To test the effect of IFNα on viral transmission, IFNα-activated DCs were pulsed with infectious HIV-1_{NL4-3} and co-cultured with primary activated $CD4^+$ T cells in the presence or absence of IFNα. As controls, $CD4^+$ T cells were pulsed with the same amount of HIV-1_{NL4-3} and co-cultured with target $CD4^+$ T cells, or directly

pulsed with cell-free virus. 2 days after co-culture, cells were stained for $CD2^+$ and intracellular p24 and revealed by FACS. Even though IFNα it's an antiviral cytokine, which *in vitro* restricts viral replication on $CD4^+$ T cells, Siglec-1-mediated viral transmission by IFNα-activated DCs in the presence of IFNα occurred. However, viral transmission by DCs was threefold decreased in the presence of IFNα (**Figure 25A**). Moreover, viral transmission by DCs was significantly more effective compared to viral transfer between $CD4^+$ T cells or cell-free virus infection of $CD4^+$ T cells (**Figure 25B**).

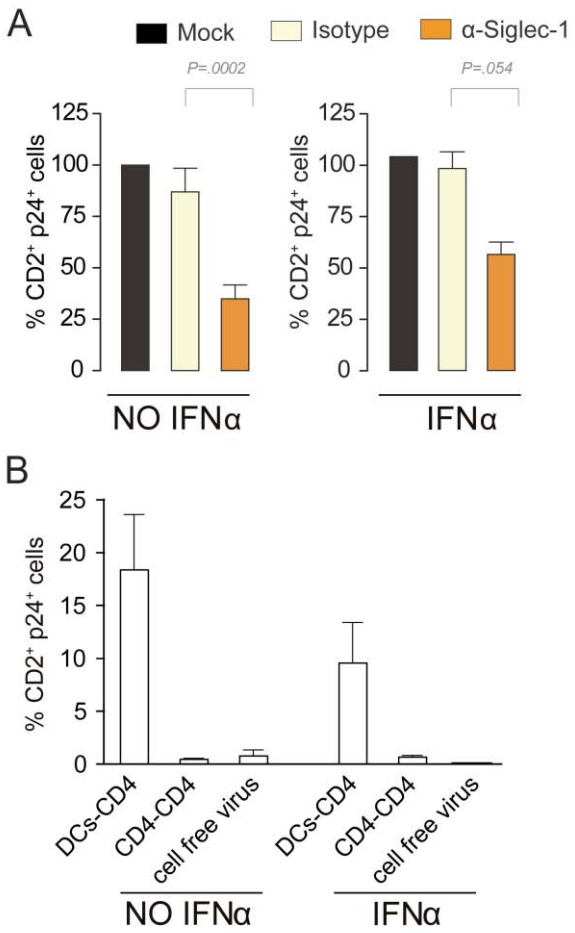


Figure 25. IFNα does not inhibit Siglec-1-mediated HIV-1 transmission to $CD4^+$ T cells. A. Relative HIV-1_{NL4-3} transmission from IFNα-activated DCs to $CD4^+$T cells in absence or presence of 10 U/ml of IFNα. Cells were stained for $CD2^+$ and intracellular p24 and analyzed by FACS at 48 h. Values are normalized to the level of HIV-1 *trans*-infected by mock-treated IFNα-activated DCs. **B.** Absolute HIV-1_{NL4-3} transmission from IFNα-activated DCs to $CD4^+$ T cells, between $CD4^+$ T cells and free virus $CD4^+$ T cell infection in absence or presence of 10 U/ml of IFNα**.** Graphs show mean values and SEM from two experiments including cells from six donors. Statistical differences were assessed with a one sample *t*-test.

In this first study we have reported that Siglec-1 on myeloid cells (i) is up-regulated upon IFNα treatment, (ii) is induced by soluble factors released by HIV-1-exposed pDCs, (iii) mediates viral uptake into a storage compartment and enhances HIV-1 *trans*-infection, and (iv) can overcome the antiviral effect of IFNα and still mediate *trans*-infection. Here, we show that immune activating signals, such as IFNα, released during HIV-1 infection could induce Siglec-1 expression on myeloid cells and contribute to HIV-1 dissemination. However, whether these *in vitro* observations reflect an *in vivo* situation remains unexplored.

Chapter 4

RESULTS II

Siglec-1 enhances HIV-1 capture and *trans*-infection on monocytes from HIV-1 infected patients

INTRODUCTION

When active viral replication bursts in HIV-1-infected individuals, peripheral blood monocytes are exposed to circulating immune activating signals present in the plasma, which can efficiently induce Siglec-1 expression on myeloid cells and most likely contribute to viral dissemination. Indeed, Siglec-1 is increased on circulating monocytes early after HIV-1 infection and its expression is maintained during disease progression [179]. Yet, whether Siglec-1 is able to capture and *trans*-infect HIV-1 *in vivo,* and consequently contribute to disease progression, is not known.

There are at least two types of immune activating signals that could induce Siglec-1 up-regulation in the plasma of HIV-1 infected individuals: IFNα and LPS. Elevated IFNα levels in plasma have been detected in untreated acutely and chronically HIV-1-infected patients [9][12][27]. Further evidences of increased IFNα bioactivity in HIV-1 untreated individuals have been demonstrated by an up-regulation of Interferon-Stimulated Genes (ISGs), such as MxA, in monocytes and DCs [181][182][183].Furthermore, during chronic HIV-1 immune activation, circulating LPS accumulates in the plasma due to the active bacterial translocation produced in the GALT of HIV-1-infected individuals [150]. Indeed, LPS signaling induces the release of type I IFN in myeloid cells [184][185]that could increase even more IFNα levels in the plasma of infected individuals.

Here, we aim to study if Siglec-1 expression is up-regulated on monocytes from untreated HIV-1-infected patients. We also elucidate if immune activating signals present in plasma from untreated HIV-1-infected patients could induce Siglec-1 expression and contribute to HIV-1 pathogenesis by modulating *trans*-infection. Our hypothesis is that increased virus-induced activating signals accumulate in the plasma of untreated HIV-1-infected individuals and up-regulate Siglec-1 expression. Under this assumption, introduction of effective antiretroviral treatment should effectively decrease activating signals and down-regulate Siglec-1 expression.

MATERIALS AND METHODS

1. Ethics statement

The institutional review board on biomedical research from Hospital Germans Trias i Pujol (HUGTIP, Barcelona) approved this study. All patients involved in this study gave their written informed consent to participate.

2. Monocytes from HIV-1-infected patients

PBMCs from HIV-1-infected patients were thawed and monocytes were isolated by positive selection using $CD14^+$ magnetic beads. A first group of HIV-1-infected subjects (n=16) was selected from a cohort of patients with samples collected before and after antiretroviral treatment. Patient's characteristics are described in **Table 3**. HIV-1-infected individuals before antiretroviral treatment were characterized by a median plasma viral load (VL) of 5 Log_{10} HIV-1 RNA copies/ml, and a median $CD4^+$ T cell number of 297.5 cells/µl. After introduction of successful antiretroviral treatment, the same individuals presented a median plasma VL of 1.7 Log_{10} HIV-1 RNA copies/ml and a median $CD4^+$ T cell number of 505 cells/µl.

Table 3. Characteristics of the HIV-1-infected individuals followed longitudinally before and after initiation of antiretroviral treatment.

PRE/POST Antiretroviral treatment	**Median (IQR)**
Number of patients	16
Sex	
Male	16
Female	0
Age (years)	36 (24–40)
Antiretroviral treatment regimen	
Non-Nucleoside Reverse Transcriptase Inhibitors	8
Protease Inhibitors	7
Integrase Inhibitors	1
Time from diagnosis to antiretroviral treatment (months)	2 (1.5-5)
Time between samples (months)	11 (7–19)
Plasma Viral Load (Log_{10} HIV-1 RNA copies/ml)	
PRE antiretroviral treatment	5 (4.4-5.5)
POST antiretroviral treatment	1.7 (1.4-1.7)
$CD4^+$ T-cell count (cells/µl)	
PRE antiretroviral treatment	297 (239–316)
POST antiretroviral treatment	505 (401–597)

A second group of HIV-1-infected subjects (n=26) was studied only before initiation of antiretroviral treatment. Patient's characteristics are described in **Table 4**. This study group was characterized by a median plasma VL of 4.3 Log_{10} HIV-1 RNA copies/ml and a median of $CD4^+$ T cell number of 338.5 cells/µl. HIV-1-negative individuals matched for age were included as seronegative controls.

Table 4. Characteristics of the HIV-1-infected individuals before initiation of antiretroviral treatment.

PRE Antiretroviral treatment	**Median** (IQR)
Number of patients	26
Sex	
Male	26
Female	0
Age (years)	36 (24–40)
Plasma Viral Load (Log_{10} HIV-1 RNA copies/ml)	4.3 (2.9-5.3)
$CD4^+$ T-cell count (cells/µl)	338.5 (285–721)

3. Siglec-1 quantitation, viral uptake and *trans*-infection assays

Siglec-1 surface expression on isolated monocytes from HIV-1-negative and HIV-1-infected individuals before or after successful antiretroviral treatment was quantified with Quantibrite beads, as described in Results I. Viral uptake and *trans*-infection assays were performed on isolated monocytes, as described in Results I.

4. Analysis of Siglec-1 induction by plasmas

Induction of Siglec-1 expression by plasmas of HIV-1-negative individuals and HIV-1-infected individuals before or after successful antiretroviral treatment was assessed on 2 x 10^5 DCs derived from HIV-1-negative donors cultured for 24 h in the presence of 2% of each respective plasma. To block Siglec-1 induction by these plasmas, carrier-free recombinant B18R protein (eBioscience) was added at 2 µg/ml. DCs were labeled with mAb 7–239 α-Siglec-1-PE and quantified by Quantibrite. Basal values of Siglec-1 in DCs non-exposed to plasma were subtracted for each sample.

5. Statistical analysis

We analyzed mean changes using a paired *t*-test, which was considered significant at $P<0.05$. Mean changes of unpaired observations were assessed using the Man Whitney *t*-test, which was considered significant at $P< 0.05$. Pearson correlation tests were used

to determine the level of association between Siglec-1 Ab binding sites per monocyte and VLP capture, HIV-1 capture, HIV-1 *trans*-infection, plasma viral load or $CD4^+$ T-cell counts from HIV-1-infected individuals. All analyses and figures were generated with the GraphPad Prism v5.0b Software.

RESULTS

1. Siglec-1 is up-regulated on monocytes from HIV-1-infected individuals, and its expression is reduced upon successful antiretroviral treatment

To explore whether Siglec-1 could be functionally important *in vivo,* we assessed Siglec-1 expression on blood monocytes from HIV-1-infected individuals before and after initiation of antiretroviral treatment (**Table 3**), and compared them to HIV-1-negative individuals. When we quantified the number of Siglec-1 Ab binding sites per monocyte *ex vivo* we found that Siglec-1 expression was significantly higher in untreated HIV-1-infected individuals compared to HIV-1-negative individuals (**Figure 26A**; 6-fold difference, $P = 0.0006$). Furthermore, Siglec-1 expression was significantly lower in monocytes isolated after antiretroviral treatment compared to monocytes isolated before antiretroviral treatment from the same patients (**Figure 26A**; 7-fold difference, $P = 0.0017$), returning to the levels showed by HIV-1-negative individuals. Next, we compared monocytes from HIV-1-infected individuals for their capacity to take up VLPs. Consistent with their higher expression of Siglec-1, cells isolated before antiretroviral treatment exhibited a higher uptake capacity for VLPs compared to cells obtained under suppressive therapy (**Figure 26B**; 19-fold difference, $P = 0.0039$). Similar results were observed for uptake of complete HIV-1 (**Figure 26C**). Consistent with the enhanced uptake, HIV-1 *trans*-infection was also higher for cells taken before antiretroviral treatment (**Figure 26D**, $P = 0.0117$). These results indicate that *in vivo,* Siglec-1 expression on peripheral blood monocytes is up-regulated by HIV-1 infection, but normalizes after effective antiretroviral treatment suppresses viral replication and the associated immune activation [9].

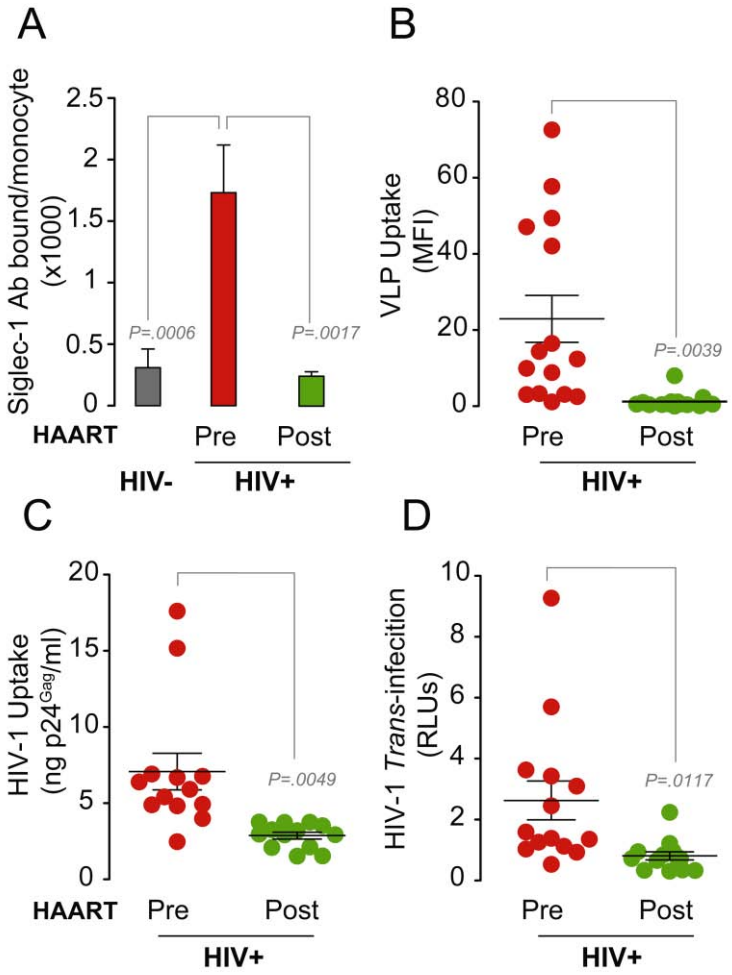


Figure 26. Siglec-1 is up-regulated on monocytes of HIV-1-infected individuals, but its expression is reduced after successful antiretroviral treatment. A. Mean number of Siglec-1 Ab binding sites per cell displayed by monocytes isolated from HIV-1-negative men and from HIV-1-infected men before or after successful antiretroviral treatment (with an initial median of 297 $CD4^+$ T-cell count and 5 $\log_{10}$ HIV-1 RNA copies/ml that changed to a median of 505 $CD4^+$ T-cell count and 1.7 $\log_{10}$ HIV-1 RNA copies/ml after treatment). Graph shows mean values and SEM from 9 HIV-1-negative individuals and 16 HIV-1-infected individuals. Man Whitney *t*-test was used to compare differences between HIV-1-negative individuals and HIV-1-infected individuals. Paired *t*-test was used to assess differences between HIV-1-infected men before or after successful antiretroviral treatment. **B.** Uptake of fluorescent VLPs by monocytes isolated from HIV-1-infected individuals before (red dots) and after (green dots) antiretroviral treatment. Cells were pulsed with VLPs for 3 h at 37°C and assessed by FACS. Graph shows mean values and SEM from 16 HIV-1-infected individuals. **C.** Uptake of $HIV\text{-}1_{NL4\text{–}3}$ by monocytes isolated from HIV-1-infected individuals before and after antiretroviral treatment. Cells were cultured with $HIV\text{-}1_{NL4\text{–}3}$ for 4 h at 37°C, washed and lysed to measure $p24^{Gag}$ by an ELISA. Graph shows mean values and SEM of the 13 individuals from which we recovered enough monocytes to perform this assay. **D.** HIV-1 transmission from monocytes isolated from HIV-1-infected individuals before and after antiretroviral treatment to a reporter $CD4^+$ cell line cultured at a ratio 5:1. Cells were pulsed with $HIV\text{-}1_{NL4\text{–}3}$ as in panel **C**. Graph shows mean values and SEM from the same individuals of panel **C**.

2. The plasma of untreated HIV-1-infected individuals stimulates Siglec-1 expression and signals via the type I IFN receptor

To assess if immune activating factors present in the plasma could trigger Siglec-1 expression on myeloid cells, we tested the capacity of such plasma to induce Siglec-1 expression on DCs derived from HIV-1-negative donors. When we quantified the number of Siglec-1 Ab binding sites per DC, we found that plasma from untreated HIV-1-infected individuals triggered Siglec-1 expression to a higher extent than plasma from HIV-1-negative individuals (**Figure 27A**). Induction of Siglec-1 expression was reduced to the level triggered by plasma from HIV-1-negative individuals when plasma from the same HIV-1-infected individuals but isolated after antiretroviral treatment was used (**Figure 17A**). This effect was mediated by signaling through the type I IFN receptor, since B18R, a soluble recombinant receptor with high affinity for type I IFNs, blocked Siglec-1 induction triggered by plasma from untreated HIV-1-infected patients **(Figure 27B**). Furthermore, addition of IFNα up-regulated Siglec-1 expression to similar levels as the plasma from untreated HIV-1-infected patients (**Figure 27B**). Moreover, plasma from those untreated HIV-1-infected individuals that displayed the highest level of Siglec-1 Ab binding sites per monocyte in peripheral blood was able to trigger Siglec-1 expression on donor DCs to a higher extent than plasma from individuals exhibiting lower levels of Siglec-1 (**Figure 27C**). Thus, the capacity to induce Siglec-1 via soluble factors in the plasma of HIV-1-infected individuals is related to Siglec-1 levels on the surface of monocytes from the respective donor, indicating that Siglec-1 expression *in vivo* is indeed regulated by soluble activation factors signaling via the type I IFN receptor.

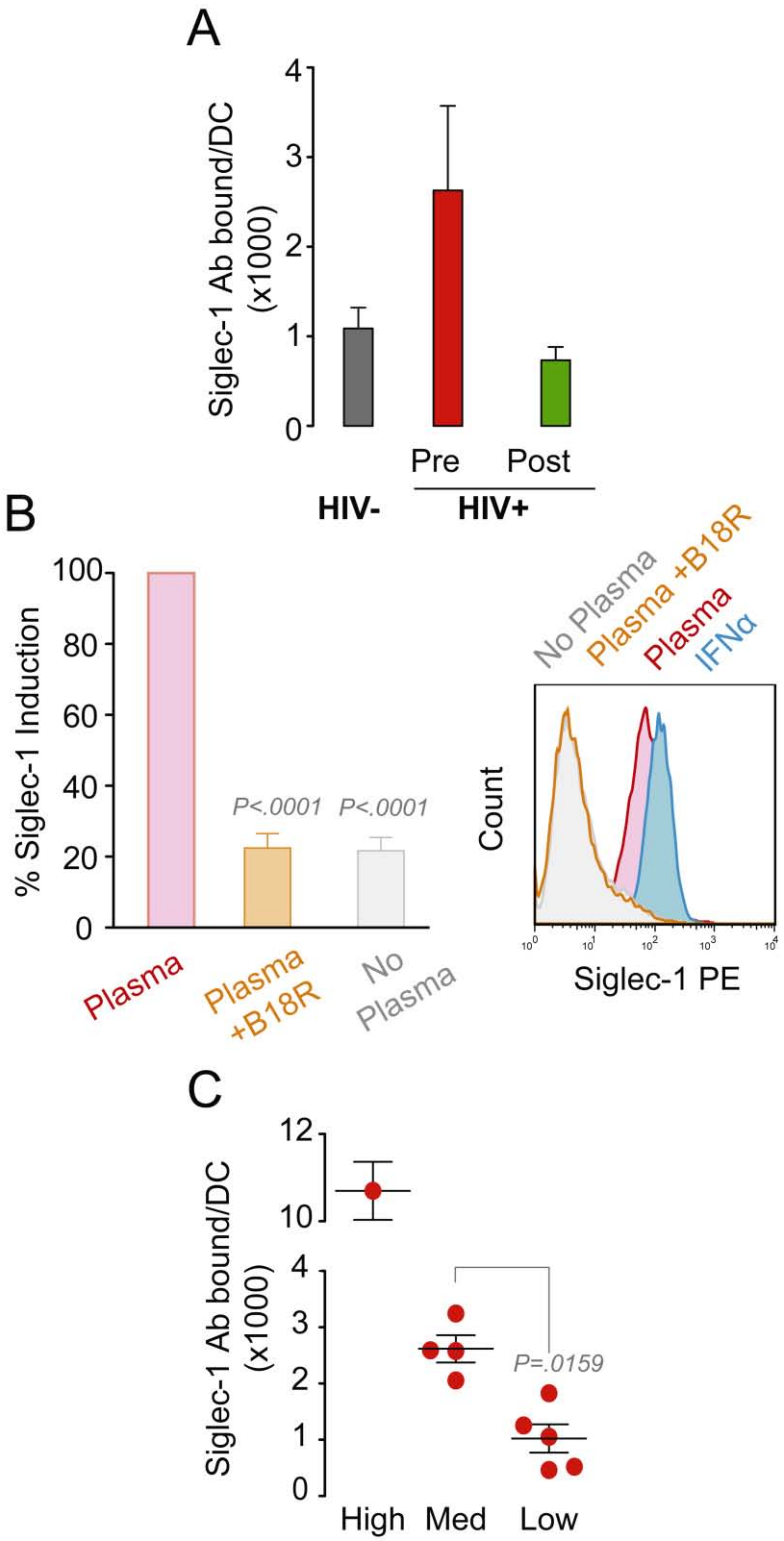


Figure 27. Caption overleaf.

Figure 27. (From previous page). The plasma of untreated HIV-1-infected individuals stimulates Siglec-1 expression and signals via type I IFN receptor. A. Mean number of Siglec-1 Ab binding sites per cell induced by the plasma of HIV-1-negative individuals and HIV-1-infected individuals before or after successful antiretroviral treatment, respectively. DCs derived from HIV-1-negative donors were cultured for 24 h in the presence of plasma and then stained for Siglec-1. Graph shows mean values and SEM of Siglec-1 induction in DCs from two donors that were tested in parallel with the plasmas from five HIV-1-negative individuals and ten HIV-1-infected individuals. **B.** Relative blockade of Siglec-1 expression by B18R, a soluble recombinant receptor with high affinity for type I IFNs, which inhibits Siglec-1 induction triggered by the plasmas of untreated HIV-1-infected individuals. DCs were cultured for 24 h with the respective plasma in the presence or absence of 2 μg/ml of B18R. Values are normalized to the level of Siglec-1 induction by plasma of mock-treated cells (set at 100%). Mean changes from 100% were assessed with a one sample *t*-test. Representative histogram also depicts IFNα-treated DCs. **C.** Mean number of Siglec-1 Ab binding sites per cell induced by the plasma of untreated HIV-1-infected individuals. DCs were cultured for 24 h in the presence of plasma collected from patients displaying the highest levels of Siglec-1 (>5500 Ab binding sites per monocyte), intermediate levels of Siglec-1 (4000–2500 Ab binding sites per monocyte) or the lowest levels of Siglec-1 (<1500 Ab binding sites per monocyte) and then stained for Siglec-1. Graph shows mean values and SEM of Siglec-1 induction in DCs from two donors that were tested in parallel with the plasmas from ten HIV-1-infected individuals. Man Whitney *t*-test was used to compare the differences between distinct plasmas to induce Siglec-1 expression.

3. Expression of Siglec-1 on monocytes correlates with clinical parameters

Focusing our analysis on antiretroviral treatment-naïve patients (**Table 4**), we found a positive correlation between Siglec-1 expression levels on isolated monocytes and i) VLP uptake (**Figure 28A**; ρ = 0.8924; *P*< 0.0001), ii) HIV-1 uptake (**Figure 28B**; ρ = 0.8069; *P* = 0.0009), and iii) HIV-1 *trans*-infection capacity (Figure 18C; ρ = 0.7836; *P* = 0.0015). In addition, Siglec-1 expression levels positively correlated with plasma viral load (**Figure 28D**; ρ = 0.6673; *P* = 0.0002). Conversely, Siglec-1 expression negatively correlated with $CD4^+$ T-cell counts (**Figure 28E**; ρ = −0.5236; *P* = 0.006).

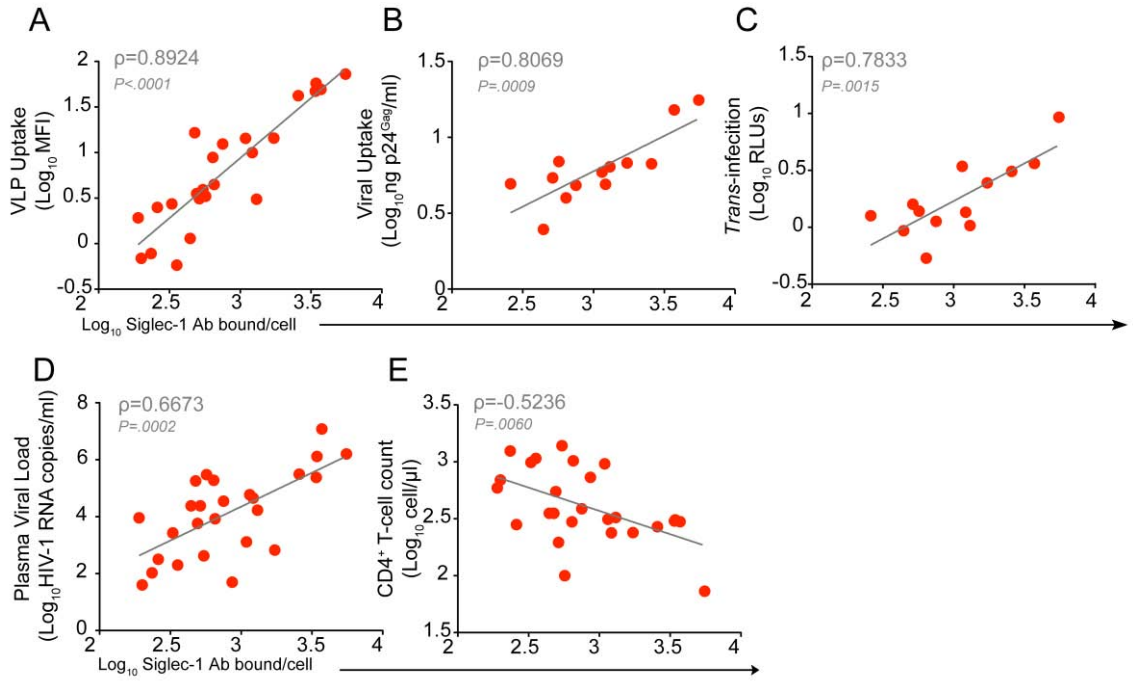


Figure 28. Expression of Siglec-1 on monocytes correlates with clinical parameters. A. Positive correlation between the mean number of Siglec-1 Ab binding sites per cell and VLP uptake of monocytes isolated from antiretroviral treatment-naïve HIV-1-infected men, with a median of 338 $CD4^+$ T-cell count and 4.3 $\log_{10}$ HIV RNA copies/ml. Graph shows the linear correlation of 26 individuals. Pearson correlation coefficient of the population is denoted by ρ. **B.** Positive correlation between the mean number of Siglec-1 Ab binding sites per cell and HIV-1 uptake of monocytes isolated from antiretroviral treatment-naïve HIV-1-infected individuals. Graph shows the linear correlation for the 13 individuals from which we recovered enough monocytes to perform this assay. **C.** Positive correlation between the mean number of Siglec-1 Ab binding sites per cell and the HIV-1 *trans*-infection capacity of monocytes isolated from antiretroviral treatment-naïve HIV-1-infected individuals. Graph shows the linear correlation for the same individuals as in panel **B**. **D.** Positive correlation between the mean number of Siglec-1 Ab binding sites per cell and the plasma viral load at the time of sample analysis of monocytes isolated from antiretroviral treatment-naïve HIV-1-infected individuals. Graph shows a linear correlation for 26 individuals. **E.** Negative correlation between the mean number of Siglec-1 Ab binding sites per cell and the $CD4^+$ T cell count at the time of sample analysis of monocytes isolated from antiretroviral treatment-naïve HIV-1-infected individuals. Graph shows a linear correlation for 26 individuals. Pearson correlation tests were used to determine the level of association between Siglec-1 Ab binding sites per monocyte and distinct parameters.

Our findings reveal that *in vivo* Siglec-1 expression is up-regulated on monocytes from HIV-1-infected individuals, but diminishes after effective antiretroviral treatment suppresses plasma viral load and virus-induced activating signals [9]. Our results are in line with previous reports showing Siglec-1 up-regulation on circulating monocytes of HIV-1-infected individuals with higher plasma viral loads [179][106]. However, assays performed here provide functional evidence that monocytes isolated directly from

HIV-1-infected individuals capture HIV-1 and *trans*-infect $CD4^+$ target cells. Moreover, we have shown that introduction of successful HAART could reduce HIV-1 immune activation, and consequently, reduce Siglec-1-mediated viral capture and *trans*-infection on monocytes. We also found that Siglec-1 expression increased with plasma viral load and decreased with $CD4^+$ T-cell counts in HIV-1 infected patients confirming that Siglec-1 could serve as a biomarker of HIV-1 disease progression. Furthermore, stimuli present in the plasma of untreated HIV-1-infected individuals induced Siglec-1 expression on myeloid cells via type I IFN receptor signaling.

After seeing the effect of immune-activating signals in peripheral blood monocytes, we next wanted to assess whether Siglec-1 could also play a role in lymphoid tissues affected by an HIV-1 driven inflammatory profile.

Chapter 5

RESULTS III

Siglec-1 is detected on myeloid cells from lymphoid tissues and enhances HIV-1 capture and *trans*-infection

INTRODUCTION

Recently, life-imaging studies have visualized for the first time how HIV-1 and murine leukemia virus (MLV) are captured by macrophages in secondary lymphoid tissues of living mice via Siglec-1-ganglioside recognition [186]. Furthermore, this study shows how MLV containing macrophages are able to form long-lived synaptic contacts and *trans*-infect MLV to target B cells *in vivo* [186]. Thus, Siglec-1-mediated *trans*-infection could be particularly relevant in lymphoid tissues, the major sites of HIV-1 replication, where myeloid cells migrate and repeatedly establish interactions with $CD4^+$ T cells, the primary targets of HIV-1 productive infection [103][124][187].

Importantly, IFNα also accumulates in lymphoid tissues of HIV-1 infected individuals; in tonsillar tissues, IFNα production is increased in HIV-1-infected individuals who progress when compared to nonprogressor or uninfected controls [166]. This increased IFNα production is mainly driven by pDCs, which are major, but not exclusive, producers of IFNα [162][188]. Indeed, during chronic HIV-1 infection, there is a redistribution of circulating pDCs to lymphoid compartments, where these cells secrete high amounts of IFNα [162][161]. Accordingly, myeloid DCs of lymphoid tissues from chronic HIV-1-infected patients present a partial activation phenotype that resembles to that of IFNα activation [187][161]. Moreover, as we have already seen, supernatant of HIV-1-exposed pDCs induces Siglec-1 expression on myeloid cells, probably by IFNα secretion, what could clearly enhances viral spread within the lymphoid tissues.

Thus, accumulation of IFNα in lymphoid tissues could induce Siglec-1 expression on myeloid cells, and consequently, contribute to viral spread throughout the course of HIV-1 infection. Here, we assessed whether IFNα stimulation could induce Siglec-1 on myeloid cells isolated from lymphoid tissues. Furthermore, we studied if Siglec-1 expression correlates with the degree of inflammation detected in lymphoid tissues using immunohistochemical analysis.

MATERIALS AND METHODS

1. Paraffinized tissues and immunoenzyme staining

1.1. Immunohistochemical analysis of lymphoid tissues from HIV-1-negative individuals

Paraffinized tonsils from HIV-1-negative individuals were provided by the tissue bank of the National Center for Tumor Diseases from Heidelberg (Germany) and approved by the ethics committee of Heidelberg University (approval No. 206/2005). Immunoenzyme staining of Siglec-1 was performed on 2 µm paraffin sections of formalin-fixed tissues as reported in [189] by Dr. E Erikson at the Institute of Medical Virology, University of Heidelberg. Antigen retrieval was achieved by steam cooking the slides in 10 mM citrate buffer (pH 6.1, Dako) for 30 min. 10% Earle's balanced salt solution (EBSS, Sigma-Aldrich) supplemented with 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.2% bovine serum albumin (BSA), and 0.1% saponin (all from Sigma-Aldrich) at pH 7.4 was used as washing and permeabilization buffer. Primary mAb dilutions with α-Siglec-1 7D2 (Novus Biologicals) were also prepared in this buffer and incubated overnight at 4°C. Slides were blocked in 15% sheep serum for 20 min and revealed by biotinylated sheep anti-mouse Ab for 30 min at RT. Immuno enzyme staining was performed with standard avidin-biotin anti-alkaline phosphatase techniques (Vectastain). Naphthol AS-biphosphate (Sigma-Aldrich) with New Fuchsin (Merck) was used as the substrate for alkaline phosphatase. Slides were viewed with an Olympus BX45 microscope. Tonsils were classified by an experienced pathologist as inflamed based on strong tissue infiltration of neutrophil granulocytes.

1.2. Immunohistochemical analysis of lymphoid tissues from an HIV-1-infected individual

Paraffinized axillary and abdominal lymph nodes from an HIV-1/Hepatitis C Virus (HCV) co-infected patient were analyzed by Dr. MT Fernández-Figueras at the Pathology Department of HUGTIP in Barcelona. For immunohistochemistry, 4 µm paraffin-embedded sections were cut, deparaffinized and rehydrated through xylene and graded alcohols to water. Antigen retrieval was done immersing the slides for 40 min

in ethylenediaminetetraacetic acid (EDTA) Buffer in a water bath at 98°C. The staining was performed using as primary mAbs α-Siglec-1 7D2, α-CD4 (Clone SP35, Ventana Medical Systems) and α-CD20cy (Clone L26, DAKO) and the Ventana Discovery XT automated stainer (Ventana Medical Systems,) with ultraView Universal DAB Detection Kit.

Of note, patient had been treated with two nucleoside reverse transcriptase inhibitors for 7 years and had stopped treatment for 4 years, when the first biopsy was performed. At that time point, HIV-1 plasma viral load was < 50 HIV-1 RNA copies/ml and $CD4^+$ T cell-count was 485 cells/µl. Patient started antiretroviral treatment again with a protease inhibitor-based regimen and had a second biopsy one year later, when HIV-1 plasma viral load was < 25 HIV-1 RNA copies/ml and $CD4^+$ T cell-count was 511 cells/µl. At the second biopsy, HCV viral load was 641.144 UI/ml.

2. Human tonsillar cells

Human tonsils were removed during tonsillectomies of healthy individuals undergoing prescribed surgery at the HUGTIP by Dr. D Guerrero.

Fresh tonsils were mechanically disrupted with a disposable sterile scalpel (Swann-Morton). RPMI-1640 medium with 10% heat-inactivated FBS was added to collect tonsillar cells that were filtered (Millex HV, 0.45 µm; Millipore) to remove tissular debris. Mononuclear tonsillar cells ($815 \times 10^6 \pm 625 \times 10^6$ cells) were isolated by Ficoll-Hypaque (Lymphoprep) gradient centrifugation.

2.1. Tonsillar myeloid cells

To isolate tonsillar myeloid cells, T and B -lymphocytes were subsequently depleted with magnetic beads against $CD3^+$ and $CD19^+$ prior blocking Fc receptors with 1 mg/ml of human Ig Gs for 20 min at RT (**Figure 29**).

Siglec-1 positive cells were isolated by sorting Siglec-1^+/$CD20^-$/$CD2^-$cells stained at 4°C for 30 min with mAbs 7–239 α-Siglec-1-PE (AbDSerotec), α-CD2-PerCP-Cy5.5 and α-CD20-PerCP-Cy5.5 (both from Becton Dickinson) prior blocking Fc receptors as described before. We obtained approximately 0.05×10^6 sorted Siglec-1 positive cells, corresponding to a 0.006% of the recovered mononuclear tonsillar cells.

Alternatively, myeloid cells were also isolated either by BDCA1 positive selection with magnetic beads (Miltenyi Biotec) or by sorting $BDCA1^+/CD20^-/CD2^-$ cells using mAbα-BDCA1-PE-Cy7, α-CD2-PerCP-Cy5.5 and α-CD20-PerCP-Cy5.5 (all from Becton Dickinson). Approximately 2 x 10^6 of isolated $BDCA1^+$ cells and 0.16 x 10^6 of sorted $BDCA1^+$ cells were obtained, corresponding to a 0.4% and 0.03% of the mononuclear tonsillar cells, respectively.

Cell sorting was performed in a FACSVantage SE (Becton Dickinson) at the Flow Citometry Unit of Institut Germans Trias I Pujol (IGTP). Samples were analyzed with a LSRII using FlowJo software to evaluate collected data.

All tonsillar cells were cultured in RPMI-1640, 10% heat-inactivated FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin and stimulated or not with 1000 U/ml IFNα for 24-48 h.

Table 5. Panel of monoclonal antibodies used to sorter Siglec-1^+ and $BDCA1^+$ cells.

Monoclonal Antibody (mAb)	Fluorochrome	Clone	Brand
BDCA1	PE-Cy7	L161	Becton Dickinson
CD20	PerCP-Cy5.5	L27	Becton Dickinson
CD2	PerCP-Cy5.5	RPA-2.10	Becton Dickinson
Siglec-1	PE	7-239	AbDSerotec

2.2. Tonsillar B lymphocytes

$CD19^+$ B lymphocytes from mononuclear tonsillar cells were positively isolated with magnetic beads (Miltenyi Biotec). Tonsillar B lymphocytes were used as a control in functional assays.

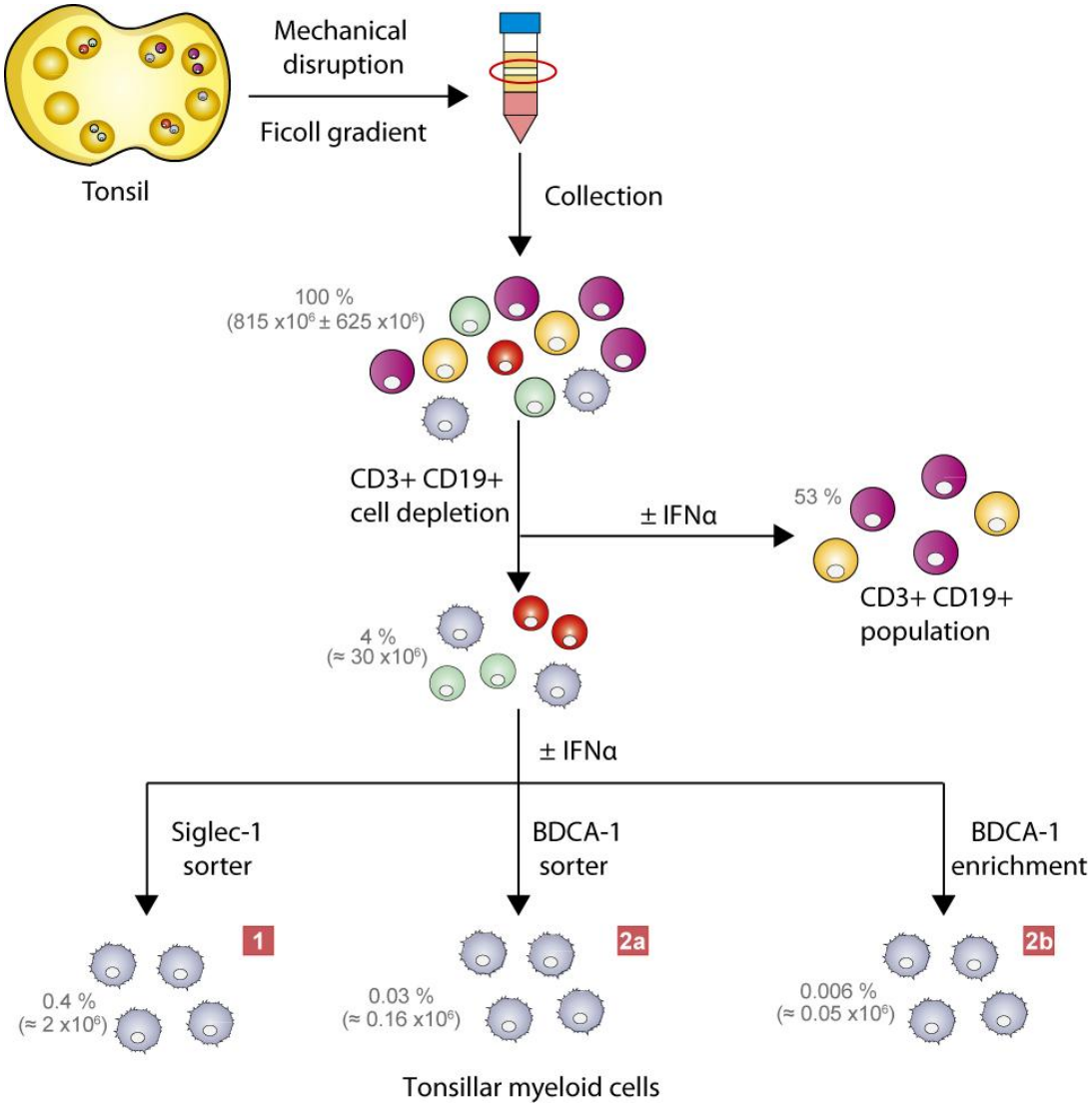


Figure 29. Workflow to enrich or isolate tonsillar myeloid cells. Isolation procedures used to obtain tonsillar myeloid cells.

3. Transcriptome RNA-seq analysis

RNA extraction from IFNα-treated DCs, macrophages and monocytes cells (1–6 x 10^6) was performed using RNeasy Mini kit (Qiagen). RNA extraction from sorted IFNα-treated Siglec-1 tonsillar cells was performed using RNeasy Micro kit (Qiagen). mRNA-Seq library preparation was done with TruSeq RNA sample prep kit, Illumina (starting with capture of polyA-containing transcripts), followed by cluster generation (TruSeq single-end cluster generation kit, Illumina) and high-throughput sequencing on Illumina HiSeq2000 at the Genomics Technology Facility, University of Lausanne. The 100 bp single-end reads obtained were cleaned before alignment as described in [190]. Cleaned reads were aligned to the human reference genome with STAR aligner [191]

using the ensembl gene GRCh37 release 70 annotation file. The number of reads per gene was quantified with HTSeq-count v.0.6.1 [192] with parameters mode = union and type = exon. We obtained an average library size of 45.072.173 uniquely mapped reads. All downstream analyses were performed taking as gene expression values the Log_{10} of the number of library size-normalized reads per kilobase of exonic sequence. A *pseudo*-count of 1 was added previous to the Log_{10} transformation to avoid NA's (impossible log transformation) and obtain a numerical value.

4. Immunophenotype of Siglec-1 tonsillar myeloid cells

To confirm transcriptome analysis, sorted tonsillar myeloid cells were characterized with a panel of different mAbs by flow cytometry upon IFNα exposure (**Table 6**). Briefly, Fc receptors were blocked with 1 mg/ml of human Ig Gs for 20 min at RT and then stained at 4°C for 30 min with the mAbs. Cells were extensively washed and fixed with 1% formaldehyde in PBS. Matched Isotype Ab controls were used. Cells were analyzed with a BD LSRII flow cytometer using DIVA and FlowJo software to evaluate collected data.

Table 6. Panel of monoclonal antibodies used to immunophenotype Siglec-1 tonsillar myeloid cells.

Monoclonal Antibody (mAb)	Fluorochrome	Clone	Brand
CD86	FITC	2331(FUN-1)	Becton Dickinson
CD83	FITC	HB15e	Becton Dickinson
DC-SIGN	PE	DCN46	Becton Dickinson
CCR7	PerCP	3D12	Biolegend
HLA-DR	PerCP	L243	Becton Dickinson
CD3	PerCP	SK7	Becton Dickinson
CD14	APC	M5E2	Becton Dickinson
CD11c	APC-Cy7	Bu15	Biolegend
HLA-DR	V450	L243	Becton Dickinson
Siglec-1	PE	7-239	AbDSerotec

5. Viral uptake and *trans*-infection assays

Viral uptake capacity of tonsillar $BDCA1^+$ isolated cells was determined by flow cytometry with a VLP uptake assay, as described in Results I, page 11. Cells were pre-

treated at 4°C for 30 min with 10 µg/ml of the functional grade mAb α-Siglec-1 7-239, Ig G1 Isotype control or left untreated. Cells were pulsed with 10 ng of VLPs for 3 h at 37°C, cells were washed with PBS and analyzed by FACS.

To reveal the fate of the captured virus, tonsillar BDCA1$^+$ sorted cells were used in confocal microscopy analysis and viral *trans*-infection assays (described in Results I, pages 14 and 13, respectively). For viral *trans*-infection assay, cells were pulsed with 970 ng HIV-1_{NL4-3} for 4 h at 37°C. Cells were washed with PBS and were co-cultured with a TZM-bl CD4$^+$ reporter cell line. *Trans*-infection was revealed 48 h later with a luciferase assay. Tonsillar CD19$^+$ B lymphocytes and tonsillar CD19$^-$/CD3$^-$/BDCA1$^-$ cells were used as negative controls.

6. Statistical analysis.

We analyzed mean changes using a paired *t*-test, which was considered significant at $P< 0.05$. Significant mean changes from 100% of the data normalized to percentages were assessed with a one sample *t*-test, considered significant at $P< 0.04$. All analyses and figures were generated with the GraphPad Prism v5.0b Software.

RESULTS

1. Siglec-1 positive cells accumulate in inflamed lymphoid tissues in areas enriched in CD4$^+$ T cells

To establish whether Siglec-1 can be detected in lymphoid tissues where myeloid cells establish continuous cell-to-cell interactions that could favor viral transmission, we first performed immunohistochemical analyses of tissues from HIV-1-negative individuals. Sections from paraffin-embedded tonsils derived from HIV-, HBV- and HCV-negative patients were classified as inflamed (n = 3) or non-inflamed (n = 3) based on histopathological criteria. Inflamed tonsils harbored on average 23-fold more Siglec-1-positive cells than non-inflamed tonsils (**Figure 30A**), indicating a clear association between the degree of immune activation and the number of cells expressing the *trans*-infection receptor.

We next assessed whether Siglec-1 expressing cells could be detected in lymphoid tissue from an HIV-1-infected individual who had lymph nodes removed before and after initiation of antiretroviral treatment. Of note, this patient also had an untreated HCV infection. Siglec-1 positive cells were detected in perivascular, sub-capsular, and perifollicular areas enriched in $CD4^+$ T cells, but mostly excluded from CD20-positive follicular zones (**Figure 30B**). A similar pattern was observed for tissue obtained before and after initiation of therapy. Although we cannot rule out the possibility that untreated HCV infection sustained Siglec-1 expression, it is also conceivable that antiretroviral drug concentrations were insufficient to fully suppress HIV-1 replication in the lymphoid tissue of this particular individual [193][194] thus sustaining IFNα production.

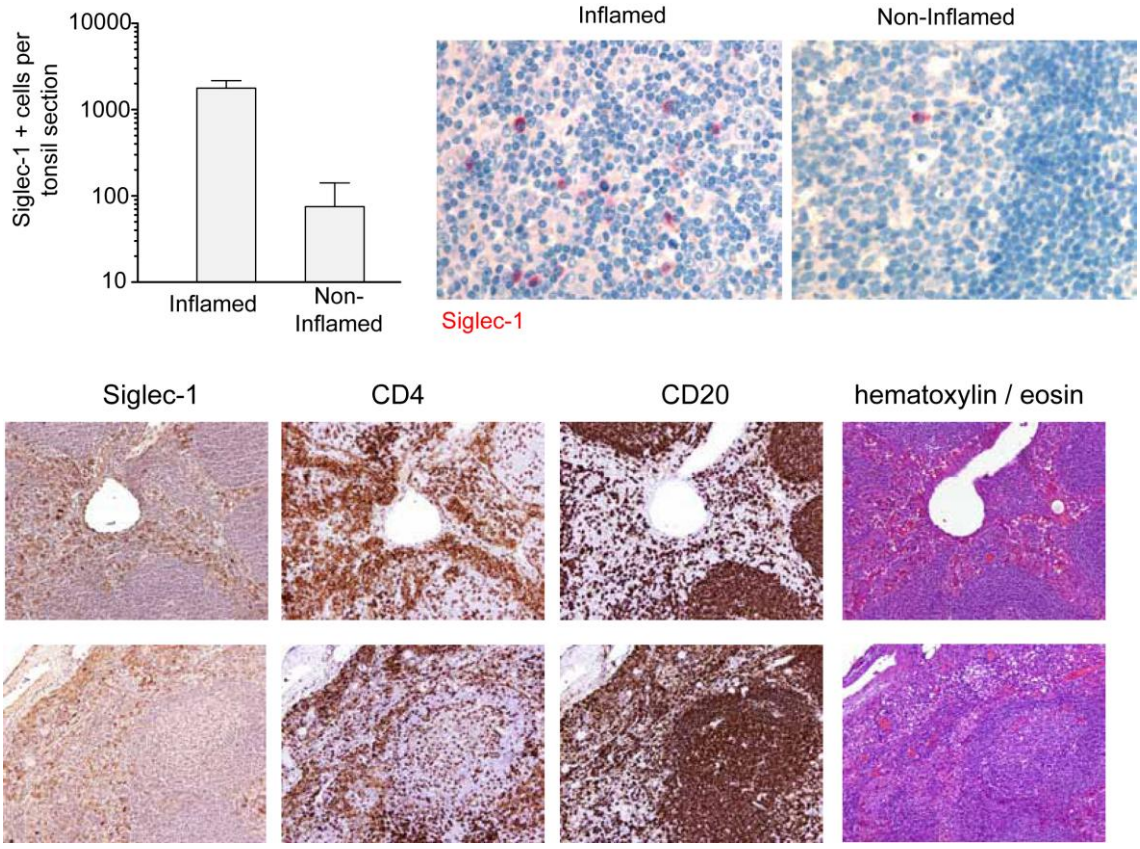


Figure 30. Siglec-1 positive cells accumulate in inflamed lymphoid tissues in areas enriched in $CD4^+$ T cells. A. Siglec-1 expression on sections of tonsils surgically removed either due to acute tonsillitis (n = 3; inflamed) or obstructive hypertrophy (n = 3; non-inflamed). Graph show mean values and SEM. Representative staining images are depicted in the right panels (magnification 40x). **B.** Staining images of Siglec-1 on lymph nodes surgically removed from an HIV-1-infected individual under antiretroviral treatment. Four continuous sections were cut and labeled independently with the following panel: anti-Siglec-1, anti-CD4, anti-CD20 or hematoxilin/eosin. Top images show perivascular distribution of Siglec-1 positive cells and bottom images display a perifollicular and subcapsular distribution.

2. Transcriptomic analyses indicate that Siglec-1 from lymphoid tissue present a unique myeloid antigen presenting cell profile

Detection of Siglec-1 on cells residing in lymphoid tissues prompted us to further characterize the role of tonsil-derived Siglec-1 positive cells in HIV-1 capture and *trans*-infection *ex vivo*. Cells were isolated from non-inflamed tonsils of HIV-1-negative individuals as depicted in **Figure 31A**. After mechanical disruption, mononuclear tonsillar cells were isolated by Ficoll-Hypaque gradient centrifugation. T- and B-lymphocytes were subsequently depleted with magnetic beads and the remaining cell fraction was cultured in the presence of IFNα or left untreated. FACS analysis revealed an up-regulation of Siglec-1 after 24 h of IFNα treatment.

We next isolated Siglec-1 positive cells by sorting of IFNα-treated tonsillar cells and performed a full transcriptome RNA-seq analysis. Results were compared to a similar RNA-seq analysis of the previous IFNα-treated myeloid cells (DCs, macrophages and monocytes), and also to $CD4^+$ T cells exposed to IFNα. Hierarchical clustering of samples on the basis of their protein coding gene expression levels revealed that sorted Siglec-1 positive tonsillar cells clustered closer to the other myeloid cells and away from $CD4^+$ T cells (**Figure 31B**). Sorted Siglec-1 positive cells shared almost 6000 protein coding genes with other myeloid cells (expressed more than 100 library size-normalized reads per kilobase of exonic sequence, **Figure 31C**), among which we found a significant enrichment of genes related to antigen-presenting functions (**Table 7**). Analysis of transcript levels of genes related to antigen-presenting functions revealed heterogeneous gene expression levels across individual myeloid cells and tonsil-derived Siglec-1 positive cells (**Figure 31D**). Siglec-1 was highly expressed in all cell types (more than 2,000 library size-normalized reads per kilobase of exonic sequence). Specific markers of tissue origin, such as CCR7 and CCL19, were found overexpressed in tonsil-derived Siglec-1 positive cells (**Figure 31D**). When we plotted a heat map-like representation based on a panel of markers recently proposed for identifying different human mononuclear phagocyte subsets [14], tonsil-derived Siglec-1 positive cells expressed most of those markers, but showed a distinctive profile (**Figure 31E**). Siglec-1 positive tonsillar cells expressed CD1c (BDCA1), CD1a and CD14 (**Figure 31E**), which are all markers found in other primary human myeloid cells isolated from

inflammatory fluids [195]. Thus, the unique pattern of tonsil-derived Siglec-1 positive cells might reflect the complexity of classifying mononuclear phagocytes under inflammatory conditions [14].Overall, transcriptomic analysis indicated that sorted Siglec-1 tonsillar cells presented a unique myeloid antigen-presenting cell profile.

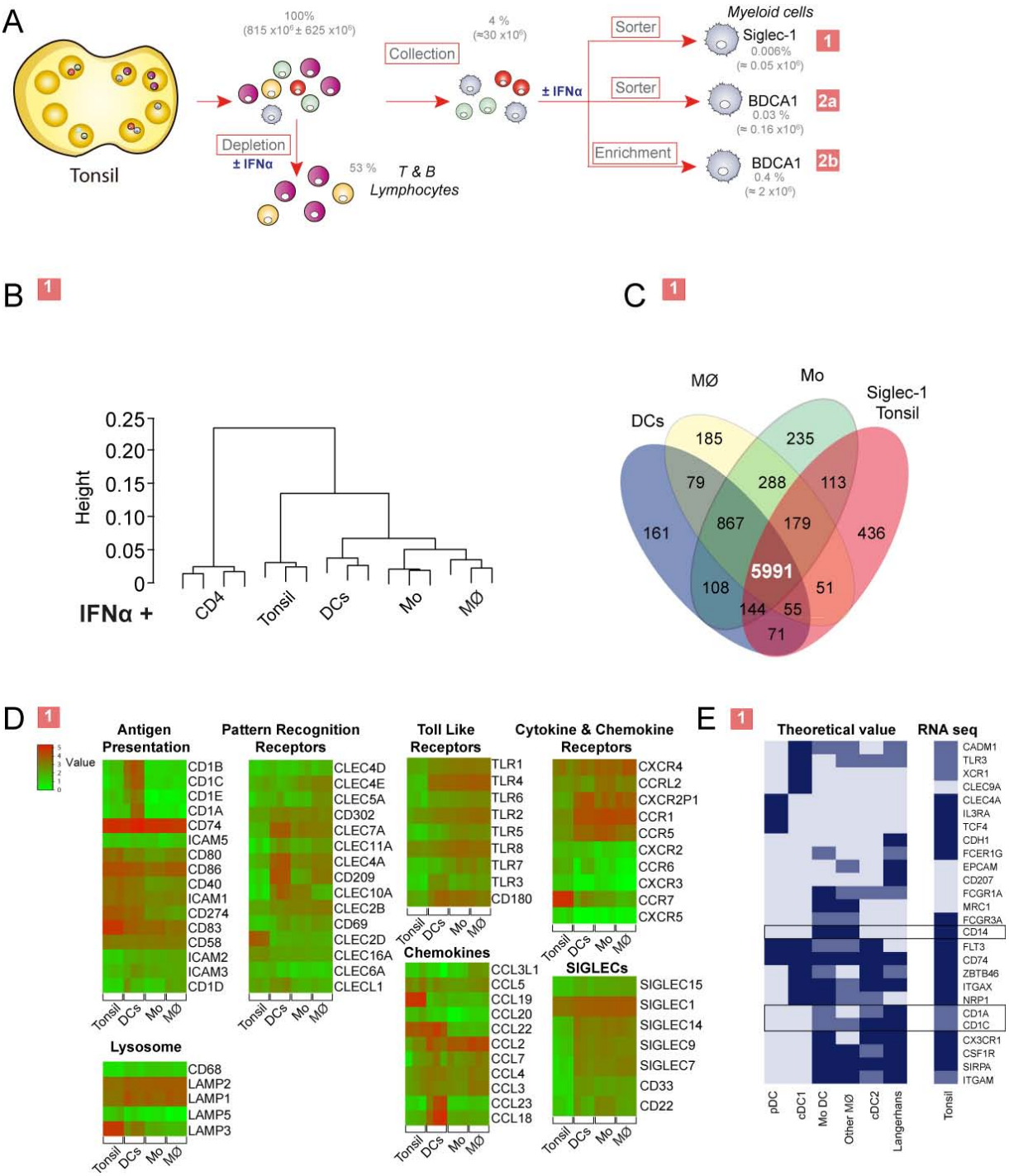


Figure 31. Caption overleaf.

Figure 31. (From previous page). Transcriptomic analyses indicate that Siglec-1 from lymphoid tissue present a unique myeloid antigen presenting cell profile. A. Workflow to enrich or isolate tonsillar myeloid cells. Red numbers on the right are used throughout the figure to identify isolation procedure. **B.** Hierarchical clustering (by Spearman correlation) of IFNα-treated sorted Siglec-1 positive tonsillar cells and primary cells based on protein-coding gene expression. **C.** Venn diagram of overlapping protein-coding genes in the indicated IFNα-treated cells. **D.** Expression heat maps of genes with antigen-presenting cell functions shared between IFNα-treated Siglec-1 sorted tonsillar cells and at least one myeloid cell type. Data in **B-D** show three donors for each cell type. **E.** Heat map-like representation of the theoretical presence/absence profile reported for markers proposed for myeloid cell classification [14] in the indicated cellular subsets (dark blue: presence; light blue: absence; blue: heterogeneous expression/unknown/unclear). Observed expression in sorted Siglec-1 tonsillar cells is also depicted (dark blue: >1.5; light blue: <1; blue: ≥1 and ≤1.5 log10). Boxes indicate markers of inflammatory myeloid cells [195].

After GO enrichment analysis, 507 GO processes were found significantly enriched, where most of the genes (3564) were related to cellular metabolic processes (GO: 0044237). Here we just summarize the enriched GO processes related to immune function, the number of genes categorized in each GO function, the actual number of genes found in the 5991 protein coding genes commonly expressed, the expected *P* value and the real *P* value obtained for the genes of interest.

Table 7. List of GO biological processes relevant for antigen-presenting cell function found significantly enriched after Bonferroni correction in the 5991 protein coding genes commonly expressed by sorted tonsil-derived Siglec-1 positive cells and the different types of myeloid cells exposed to IFNα.

GO biological process	*Genes in GO process*	*Genes found*	*Expected*	*P-value*
Immune system process (GO:0002376)	1875	791	5,10E + 05	1,38E-27
Regulation of immune system process (GO:0002682)	1090	439	2,96E + 05	3,63E-09
Positive regulation of immune system process (GO:0002684)	636	273	1,73E + 05	2,06E-06
Defense response (GO:0006952)	1256	490	3,42E + 05	1,30E-08
Regulation of defense response (GO:0031347)	502	218	1,37E + 05	1,69E-04
Immune response (GO:0006955)	1153	476	3,14E + 05	3,06E-12
Regulation of immune response (GO:0050776)	712	303	1,94E + 05	2,89E-07
Positive regulation of immune response (GO:0050778)	428	200	1,16E + 05	2,52E-06
Activation of immune response (GO:0002253)	344	175	9,35E + 04	7,68E-08
Immune response-activating signal transduction (GO:0002757)	296	165	8,05E + 04	2,15E-10
Immune response-regulating signaling pathway (GO:0002764)	403	202	1,10E + 05	3,18E-09
Immune effectorprocess (GO:0002252)	399	194	1,09E + 05	1,82E-07
Response to virus (GO:0009615)	250	129	6,80E + 04	7,85E-05

GO biological process	*Genes in GO process*	*Genes found*	*Expected*	*P-value*
Innate immune response (GO:0045087)	773	362	2,10E + 05	6,72E-16
Regulation of innate immune response (GO:0045088)	246	141	6,69E + 04	4,76E-09
Positive regulation of innate immune response (GO:0045089)	178	106	4,84E + 04	1,63E-06
Activation of innate immune response (GO:0002218)	148	94	4,02E + 04	1,04E-06
Innate immune response-activating signal transduction (GO:0002758)	141	91	3,83E + 04	1,05E-06
Antigen processing and presentation (GO:0019882)	222	142	6,04E + 04	5,93E-13
Of peptideantigen (GO:0048002)	186	126	5,06E + 04	8,41E-13
Of peptide antigen via MHC class I (GO:0002474)	101	81	2,75E + 04	3,02E-10
Of exogenousantigen (GO:0019884)	172	119	4,68E + 04	1,77E-12
Of exogenous peptide antigen (GO:0002478)	170	117	4,62E + 04	5,08E-12
Of exogenous peptide antigen via MHC class I (GO:0042590)	79	64	2,15E + 04	3,07E-07
Of exogenous peptide antigen via MHC class I, TAP-dependent (GO:0002479)	75	61	2,04E + 04	9,76E-07
Pattern recognition receptor signaling pathway (GO:0002221)	138	89	3,75E + 04	1,97E-06
Toll-like receptor signaling pathway (GO:0002224)	120	80	3,26E + 04	5,80E-06
Toll-like receptor 3 signaling pathway (GO:0034138)	79	57	2,15E + 04	5,06E-04
Toll-like receptor 4 signaling pathway (GO:0034142)	95	63	2,58E + 04	1,52E-03
Toll-like receptor 9 signaling pathway (GO:0034162)	73	52	1,99E + 04	4,80E-03
TRIF-dependent toll-like receptor signaling pathway (GO:0035666)	76	54	2,07E + 04	2,56E-03
Cellular response to cytokine stimulus (GO:0071345)	457	218	1,24E + 05	3,25E-08
Cytokine-mediated signaling pathway (GO:0019221)	339	169	9,22E + 04	1,08E-06
Regulation of type I interferon production (GO:0032479)	106	78	2,88E + 04	9,02E-08
Positive regulation of type I interferon production (GO:0032481)	75	56	2,04E + 04	2,13E-04

3. Siglec-1 mediates HIV-1 capture and is stored in Siglec-1 positive compartments by myeloid cells isolated from lymphoid tissue

Sorted Siglec-1 positive cells from IFNα-treated tonsils co-stained with several myeloid markers that had been identified in the transcriptomic analysis, including BDCA1, CD11c, HLA-DR, CCR7 and CD86 (**Figure 32A**, top panels). However, sorted Siglec-1 positive cells could not be employed in functional assays, since mAbs against Siglec-1 block HIV-1 capture (**Figure 21C; Results I**). When we sorted BDCA1-positive cells from IFNα-treated tonsillar cells, they also stained positive for Siglec-1, CD11c, HLA-DR, CCR7 and CD86 (**Figure 32A**, bottom panels), indicating that this population had a

comparable phenotype to that exhibited by Siglec-1 positive cells and could be used for functional assays.

Viral uptake experiments performed with IFNα-treated BDCA1-positive tonsillar cells demonstrated a higher VLP capture capacity when compared to mock-treated BDCA1-positive cells (**Figure 32B**), and was specifically inhibited by pre-treatment with an anti-Siglec-1 mAb (**Figure 32B).** Of note, neither the BDCA1-negative cell population nor B cells, which express BDCA1 and could thus be present in the BDCA1-positive cell fraction, were able to up-regulate VLP uptake after IFNα treatment (**Figure 32C**). In order to investigate HIV-1 trafficking in IFNα-treated BDCA1-positive cells, we added fluorescent HIV-$1_{NL4\text{-}3\text{-}Cherry}$ for 4 h at 37°C and subsequently stained cells with an anti-Siglec-1 mAb (**Figure 32D**). Confocal microscopy indicated that most of these BDCA1-positive cells accumulated HIV-$1_{NL4\text{-}3\text{-}Cherry}$ within a sac-like compartment enriched in Siglec-1, as previously observed for DCs and monocytes (**Figure 32E**).

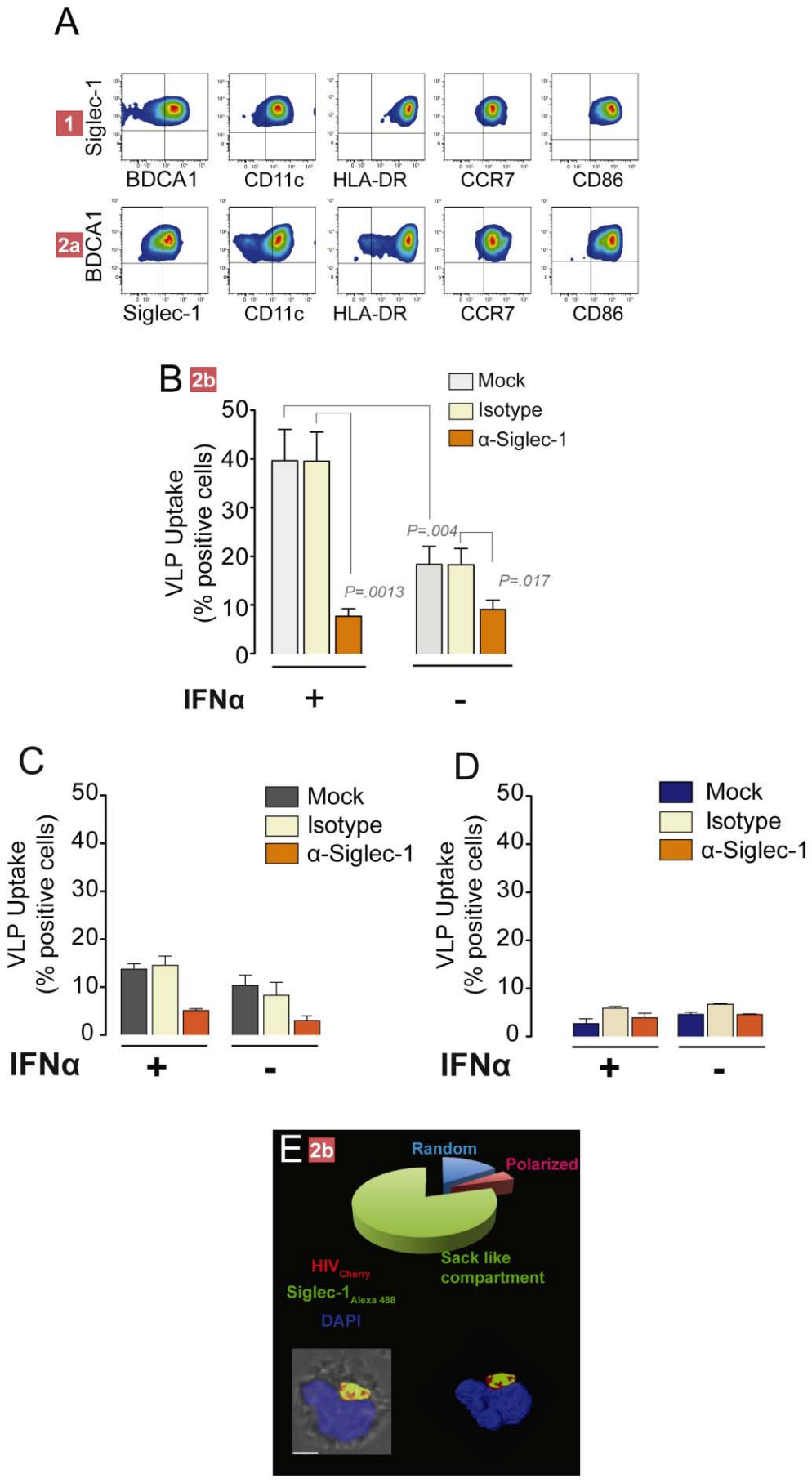


Figure 32. Caption overleaf.

Figure 32. (From previous page).Siglec-1 mediates HIV-1 capture and is stored in Siglec-1 positive compartments by myeloid cells isolated from lymphoid tissue. A. Representative FACS staining of selected myeloid markers in sorted Siglec-1 positive cells (top) and BDCA1-positive cells (bottom). **B.** Uptake of VLPs by BDCA1-positive tonsillar cells exposed to IFNα or left untreated. Cells were pre-incubated with indicated mAbs. Mean values and SEM from three experiments include cells from eight donors, assessed with a paired *t*-test. **C.**Uptake of VLPs by $CD19^{-}/CD3^{-}/BDCA1^{-}$-tonsillar cells previously exposed to 1000 U/ml of IFNα or left untreated for 48 h. Cells were pre-incubated with 10 µg/ml of the indicated mAbs before VLP exposure for 3 h at 37°C. Data show mean values and SEM from 2 experiments including cells from 5 donors. **D.** Uptake of VLPs by $CD19^{+}$ tonsillar cells previously exposed to 1000 U/ml of IFNα or left untreated for 48 h. Cells were pre-incubated with 10 µg/ml of the indicated mAbs before VLP exposure for 3 h at 37°C. Data show mean values and SEM including cells from 2 donors. **E.** Confocal microscopy of IFNα-treated BDCA1-positive tonsillar cells (n = 2) pulsed with $HIV\text{-}1_{NL4\text{-}3\text{-}Cherry}$ and stained for Siglec-1. Representative viral pattern (maximum fluorescence intensity of three channels and bright field).

4. Siglec-1 enhances *trans*-infection by myeloid cells isolated from lymphoid tissue

Finally, to work with highly purified cell populations, we sorted $BDCA1^{+}CD2^{-}CD20^{-}$-tonsillar cells cultured in the presence of IFNα and assessed Siglec-1 involvement in HIV-1 *trans*-infection. IFNα-activated BDCA1-positive cells pre-treated with isotype control or specific mAb were exposed to HIV-1 for 4 h at 37°C, extensively washed and co-cultured with a $CD4^{+}$ reporter cell line for 2 days (**Figure 33**). *Trans*-infection was readily observed and was specifically inhibited by pre-treatment with a mAb against Siglec-1 (**Figure 33**). These results indicated that *ex vivo*, activation of myeloid cells from tonsils with IFNα leads to Siglec1-dependent enhanced HIV-1 capture and *trans*-infection, supporting a potential role of Siglec-1 as an important molecule that could contribute to viral capture and *trans*-infection within lymphoid tissues in HIV-1-infected individuals.

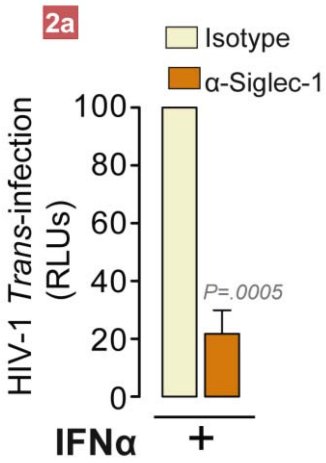


Figure 33. Siglec-1 enhances *trans*-infection by myeloid cells isolated from lymphoid tissue. HIV-1 transmission from IFNα-treated sorted BDCA1-positive tonsillar cells to a reporter $CD4^+$ cell line. Cells were pre-incubated with indicated mAbs. Values are normalized to the level of HIV-1 *trans*-infected by isotype-treated cells. Mean values and SEM from two experiments include mixed cells from six donors, assessed with a one sample *t*-test.

Detection of Siglec-1 within lymphoid tissues suggests that this receptor could mediate HIV-1 capture and transmission in these compartments. Lymphoid tissues are the perfect scenarios to fuel novel infections, since they are major sites of HIV-1 replication [196], where plasmacytoid and myeloid cells accumulate during the course of HIV-1 infection [161][187] and where IFNα is detected in lymph nodes of HIV-1-infected individuals [120][162]. Functional assays performed here with myeloid cells isolated from tonsils and activated with IFNα (to mimic the immune activation state driven by HIV-1 infection in the lymphoid tissues), identified Siglec-1 as a key receptor involved in viral capture and transmission. Accordingly to this, Siglec-1 positive tonsillar cells were able to capture and store HIV-1 in a Siglec-1 positive compartment as already seen in monocytes and monocyte-derived DCs.

Chapter 6

RESULTS IV

Small inhibitory compounds or antibodies against Siglec-1 peptides do not block viral capture

INTRODUCTION

We have demonstrated that Siglec-1 expression mediates viral transmission not only by circulating monocytes, but also by myeloid cells from lymphoid tissues. Our results strongly suggest that Siglec-1 mediated viral *trans*-infection is an important mechanism of cell-to-cell viral transmission that HIV-1 uses to evade the immune system and infect new $CD4^+$ T cells. Furthermore, Siglec-1 viral spread could contribute to the maintenance of ongoing viral replication, the settlement of viral reservoirs and the formation of anatomical sanctuaries within lymphoid tissues, hampering the efficacy of eradication strategies. Despite the prominent role that cell-to-cell viral transmission plays in HIV-1 pathogenesis [104][197], no current antiviral treatment targets Siglec-1 viral transfer ability. Therefore, specific inhibitors against Siglec-1 could pose a novel therapeutic strategy to block cell-to-cell viral transmission. We envisage this treatment as a complement to current antiviral treatments; anti-Siglec-1 compounds should therefore be administered together with the existent HAART to synergistically reduce viral replication and limit viral reservoirs.

Here we aimed to identify a safe and potent inhibitory compound to use in a novel therapeutic approach that could enhance current treatment options and limit ongoing viral replication. Basic knowledge gathered in this preliminary screening will set up the basis for developing a new generation of compounds and/or monoclonal antibodies designed to block viral transmission mediated by Siglec-1 that could be tested in animal models for future clinical use. Thus, we present a rational approach to screen between different inhibitory agents and select the most promising candidate using a cell culture model that could be easily implemented for high throughput screening.

We focused on two main types of molecules available for the clinics: small-molecule agents and therapeutic monoclonal antibodies (mAbs). Successful Siglec-1 blockage will most likely require high binding affinity and specificity. Nevertheless, Siglec-1 interactions with its natural ligand, syalillactose, present very low binding affinity [134]. Clustering of ligands is needed to originate stable interactions with Siglec-1 receptor [198][199]. Thus, using small syalillactose-like molecules could result in an insufficient binding to Siglec-1. The design of a multivalent structure presenting several

syalillactose-like moieties could increase binding avidity and block Siglec-1. These two approaches could be cost-effective and safer than using mAbs [200], on the other hand, the efficacy of commercial available mAbs against Siglec-1 in preventing viral capture is highly effective *in vitro* [135]. Indeed, commercial mouse mAbs, that has been produced by mice immunization with human Siglec-1, successfully blocks viral capture [135], most likely due to steric hindrance that prevents viral recognition.

Since both mAbs and small molecules present benefits and drawbacks from a clinical point of view, in this study we pursued to develop both syalillactose-like small compounds (in monovalent and multivalent formulations) and specific human mAbs against the V-Set domain that could effectively block viral capture and cell-to-cell transmission mediated by Siglec-1.

MATERIALS AND METHODS

1. Siglec-1 expressing cell line

Raji B-lymphocyte cell line (kindly provided by Dr. Y. Van Kooyke) was used to develop a Siglec-1 expressing cell line. We performed a nuclear transfection with a Siglec-1 plasmid (pCMV6 Siglec-1 Myc-DDK; Origene) using Amaxa nucleofector (Lonza) as recommended by the manufacturer. Upon transfection, cells were maintained for three months and passed twice a week using RPMI-1640 medium containing 10% FBS, 100 U/ml of penicillin, 100 µg/ml of streptomicin and 1 mg/ml of geneticin. Siglec-1 positive cells were sorted in a FACSVantage SE at the Flow Cytometry Unit of IGTP. In each experiment Siglec-1 expression was analysed by flow cytometry in order to assure that Siglec-1 expressing levels were optimal to perform the *in vitro* assays.

2. Screening for new Siglec-1 blocking syalillactose-like compounds

2.1. *In sillico* screening of syalillactose-like compounds

In collaboration with Dr. R. Pascual and Dr. J. M. Vela from ESTEVE, a 3D structure model of the human V-set domain of Siglec-1 was constructed, built by homology to the high-resolution murine crystal structures available [134]. Modeling software was used to design and compare the prototypic human Siglec-1 protein structure with the

murine crystal structures of the protein, specially focusing on the sialyllactose-binding domain. A pharmacophore model was also designed to virtually assess ligand binding to human Siglec-1 and perform an *in silico* screen of a drug database comprising more than 6 million unique-structure compounds. This pharmacophore allowed efficient selection by docking techniques of compounds whose chemical structure resembles to that of sialyllactose, which binds to the V-set domain of Siglec-1. Most promising compounds were ordered, synthesized (**Table 8**) and tested for their efficacy in functional blocking assays.

Table 8. Chemical characteristics of the sialyllactose-like compounds identified *in silico*.

ESTEVE_ID	Molecular Mass	Log(S, Mol/l)_Sol_In_PW	pH_Sol_In_PW
EST0065571.U	467,42	-1,572	1,75
EST0065572.U	537,74	-2,477	2,56
EST0065573	430,36	-1,462	1,62
EST0065574	385,37	-1,115	1,45
EST0065606	399,47	-2,738	6,57
EST0065607	539,54	-3,519	3,67
EST0065608	537,56	-3,786	3,88
EST0065609	534,52	-3,593	3,71
EST0065610	293,30	-3,136	3,72
EST0065611	396,40	-2,918	3,34
EST0065612	371,43	-2,635	7,37
EST0065864	226,19	-0,979	2,75
EST0065865.A	315,44	-2,684	7,5
EST0065866	299,30	-2,063	3,32
EST0065867	333,32	-3,401	3,58
EST0065868	255,23	-1,39	5,33
EST0065869	400,36	-1,135	1,15
EST0065870	189,17	-1,036	2,16
EST0065871	293,27	0,352	1,5
EST0065872	417,48	-1,535	2,61
EST0065873	534,47	-3,01	3,88
EST0065874	471,54	-2,338	3,55
EST0065875	504,44	-2,917	3,84

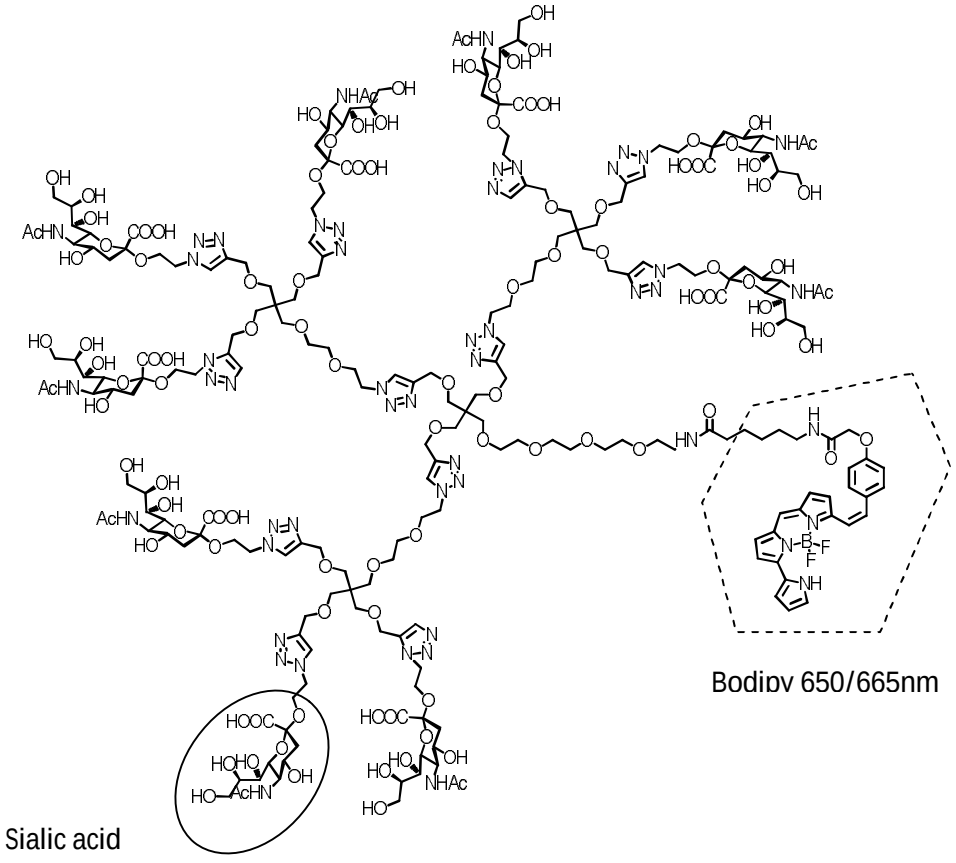
2.2. Blockade of VLP capture with sialyllactose-like compounds

To assess whether sialyllactose-like compounds could efficiently block Siglec-1 and diminish viral capture, we performed a viral capture assay using VLPs. 2 x 10^5 Raji-Siglec-1 cells were pre-incubated at 4°C for 30 min with the most promising compounds (n=23; **Table 8**) at 10 mM, 0.1 mM, and 1 µM. As a negative control, Raji-Siglec-5 expressing cells were also tested. As a positive control, we included 3'-Sialyllactose at 10 mM (Carbosynth). Raji-Siglec-1 cells were pulsed with 159 pg of VLPs per 2 x 10^5 cells for 2 h at 37°C in a final volume of 100 µl. After washing, Raji-Siglec-1 cells were acquired in a FACSCalibur. Samples were analyzed using CellQuest and FlowJo software.

3. Blockade of VLP capture with syalillactose-multivalent compounds

Our collaborators Dr. J. J. Reina and Dr. J. Rojo at the Instituto de Investigaciones Químicas (IIQ - CSIC), synthetized multivalent structures bearing up to 9 sialic acid moieties or 3 sialyllactose moieties. These structures are known as glycodendrons, and were labeled with fluorescent Bodipy 650/665 nm (**Figure 34**).

A



B

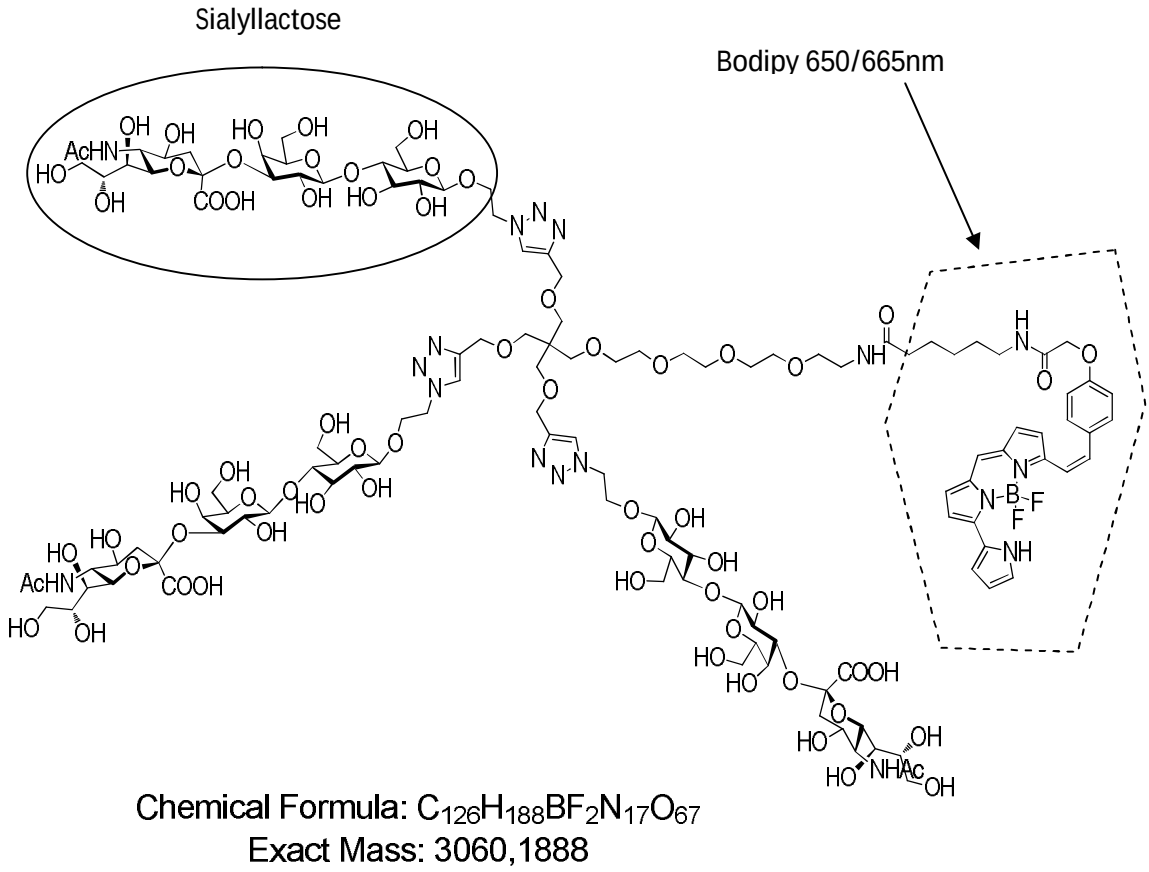


Figure 34. Glycodendron structures labeled with fluorescent Bodipy 650/665nm. A. Glycodendron structure composed of 9 sialic acids moieties. **B.** Glycodendron structure composed of 3 sialyllactose moieties.

3.1. Blockade of VLP capture with sialic acid containing multivalent glycodendrons

0.2 x 10^6 LPS mDCs were pre-incubated for 30 min at 4°C in the presence or absence of the sialic acid or sialyllactose glycodendron Bodipy 650/665nm at 1 μM, 10 μM and 100 μM. Cells were then pulsed with 150 pg of VLPs for 30 min at 37°C in the presence of the glycodendrons. After washing, mDCs were acquired in a FACSCalibur. Samples were analyzed using CellQuest and FlowJo software.

To determine whether glycodendrons specifically recognize Siglec-1 receptor, LPS mDCs were pre-treated with 10 μg/ml of mAb 7-239 α-Siglec-1 or matched Isotype control for 30 min at 4°C. Cells were then pulsed with increasing concentrations of the glycodendrons (1 μM, 10 μM and 100 μM) for 30 min at 37°C. After washing, mDCs were acquired in a FACSCalibur (BD). Samples were analyzed using CellQuest and FlowJo software.

4. Mice immunization with Siglec-1 immunogenic peptides

These experiments were performed in collaboration with Dr. M. García-Gallo and Dr. L. Kremer at the Protein Unit Tools of Centro Nacional Biotecnología (CNB).

4.1. Peptide design

To maximize the possibility of generating mAb that specifically recognize the epitope bound by HIV-1, we selected three immunogenic regions of the Siglec-1 V-set domain based on i) the differences among human and mouse protein alignments (**Figure 35**); ii) the physicochemical and structural characteristics of putative sialyllactose binding sites described in the literature [134]; and iii) the critical amino acids required for Siglec-1 activity [134]. Peptides of 16 (Sig20), 15 (Sig113) and 13 amino acids (Sig122) were synthesized and covalently coupled to the carrier protein KLH (Keyhole limpet hemocyanin).

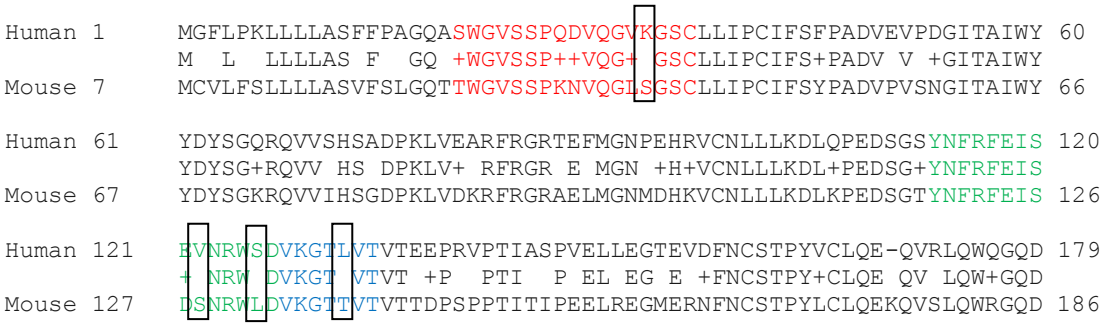


Figure 35. Differences between the amino acid alignments of the human and the mouse V-set domain that highlight potential peptide candidates for mice immunization. Non-conserved changes between species are highlighted in boxes. The amino acid sequences of the selected peptides used for immunization are shown in color: Sig20 (red), Sig113 (green) and Sig122 (blue).

4.2. Mice immunization schedule

Five female mice BALB/c were immunized 3 times with Sig20, Sig113 or Sig122 immunogens. Mice received three subcutaneous injections in a 30-day interval of each peptide covalently coupled to KLH (**Figure 36**): at day 0, 30, and 60, mice were subcutaneously injected into the dorsal area with the protein solution (30 µg in PBS) as an emulsion in Freund's Adjuvant, in a final volume of 300 µl per animal. At day 70 to 74, blood was extracted to analyze serum responses.

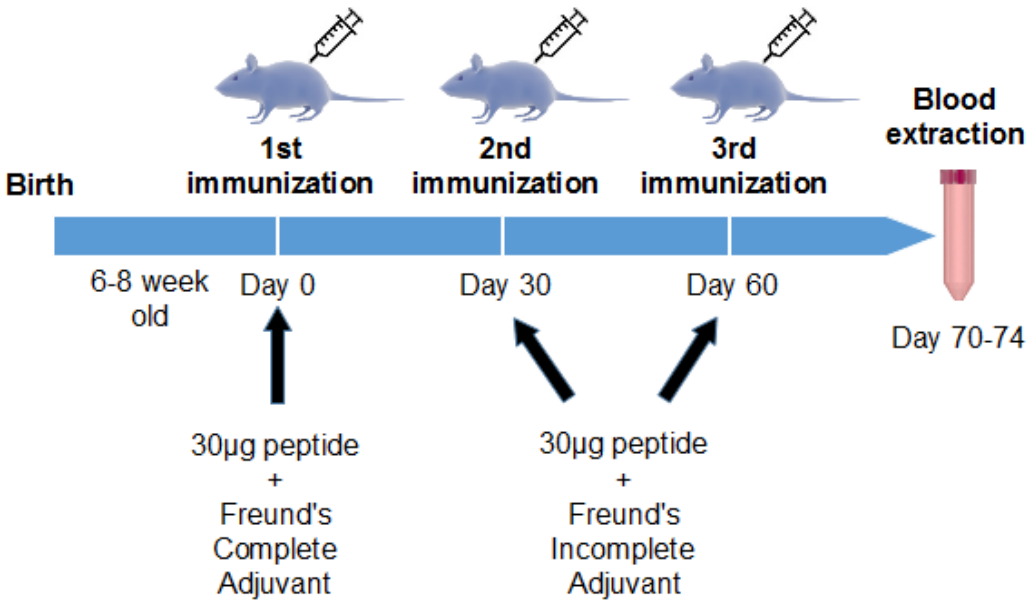


Figure 36. Mice immunization schedule. At day 0, 30, and 60 mice were subcutaneously injected into the dorsal area with the protein solution (30 µg in PBS) as an emulsion in Freund's Adjuvant, in a final volume of 300 µl per animal. At day 70 to 74, blood was extracted to analyze serum responses.

Blood samples were collected from immunized mice to quantify the presence of antibodies in the sera. Collected samples were maintained at room temperature for 1 h to allow coagulation and maintained at 4°C for 12-14 h. Blood was centrifuged to separate and collect the serum.

4.3. ELISA of sera from immunized mice

To determine the presence of specific antibodies in sera we performed an ELISA with synthetic linear peptides Sig20, Sig113 and Sig122 covered wells. Sera were diluted (from 1/300 to 1/72900) to determine the titer, expressed as half the maximal signal. Sera from mice immunized with a non-related peptide was used as a negative control.

96-well plates MaxiSorp (Nunc) were covered with 100 µl of 1 µg/ml of synthetic peptides (Sig20, Sig113 or Sig122) in PBS, and incubated at 4°C for 18 h. After several washes, unspecific binding sites were blocked with 0.5% BSA for 1 h at RT. After washing steps, dilutions of immune sera and control sera were added and incubated 1 h at RT. Upon extensive washing, antibody from goat anti-mouse Ig G coupled with horseradish peroxidase (Goat anti-mouse-HRP, Dako) was incubated at 1:1500 dilution.

Antigen reactivity was developed with 0.4 mg/ml of ortho-Phenylenediamine (OPD, o-Phenylenediamine dihydrochloride, Sigma-Aldrich) in citrate buffer 0.05 M pH=5 with 0.015% H_2O_2. Reaction was stopped with 2.5 M H_2SO_4 and absorbance measured at 490 nm in a plate reader (680Bio-rad). As positive controls, 1 µg/ml of the mAbs 7D2 (Novus Biologicals) and 7-239 (AbD Serotec) α-Siglec-1 were used.

4.4. Blockade of VLP capture with sera from immunized mice

2 x 10^5 Raji-Siglec-1 cells were pre-incubated at 4°C for 30 min with distinct sera from immunized mice diluted at 1/50, 1/150, 1/450 and 1/1350. As a blocking control, we included the mAb α-Siglec-1 7-239 (AbD Serotec) at 10 µg/ml. As a negative control, we included a non-related serum. Raji-Siglec-1 cells were pulsed with 159 pg VLPs per 2 x 10^5 cells for 2 h at 37°C. After extensive washing, Raji-Siglec-1 were acquired and analyzed by FACS.

4.5. Raji-Siglec-1 cell staining with sera from immunized mice

A total of 2.5 x 10^5 cells were washed with 0.5% BSA, 1% FBS and 0.1% sodium azide in PBS (PBSst). To block unspecific binding, cells were pre-incubated in PBSst with 40 µg/ml of Ig G from rat serum (Sigma-Aldrich) in a final volume of 100 µl for 30 min at 4°C. Cells were incubated with the primary unlabeled Ab (sera from immunized mice or commercial mAbs against Siglec-1) for 30 min at 4°C, washed and incubated with goat F(ab')2 anti-mouse IgG (H+L) labeled with PE (Cell Lab). Isotype primary Ab was used as a control. Samples were analyzed by FACS in a Cytomics FC500 (Beckman Coulter) and analyzed with FlowJo software.

5. Design and production of a recombinant V-set domain protein of Siglec-1

These experiments were performed in the laboratory of Dr. M. Lorizate during a short stay in the Universidad del País Vasco (UPV/EHU). We constructed a plasmid encoding the V-set domain of Siglec-1 coupled to a histidine tag. V-Set sequence was amplified by Polimerase Chain Reaction (PCR, Clone *Pfu* DNA polymerase kit; Agilent Technologies) from plasmid pCMV6 Siglec-1 Myc-DDK (Origene), following manufacturer's protocol. We constructed another plasmid by amplifying the V-set

domain plus the Siglec-1 leader peptide. Forward and reverse primers (Integrated DNA Technologies) included an AgeI and BamHI restriction enzyme sites, respectively (**Figure 37A**). Reverse primer included a linker composed of Gly-Gly-Gly-Gly-Gly-Ser-Gly at the C terminus of Siglec-1 before the histidine tag. Oligos sequences used were: Forward-without-leader (nucleotides 109-145) 5' GAG TTA ACC GGT ATG TCA TGG GGC GTC TCC AGT CCC 3', Forward-with-leader (nucleotides 54-87) 5' GAG TTA ACC GGT ATG GGC TTC TTG CCC AAG CTT 3', Reverse (nucleotides 451-487) 5' TATTTA GGA TCC CCC GCC CCC CCC TGT TAC TGT GAC CAA GGT GCC 3'. The thermocycling conditions were: 95°C 2 min, 30 cycles at 95°C 30 sec, 53°C 30 sec and 72°C 1 min, and a final cycle of 72°C 10 min. PCR-amplified fragments were revealed in a 1% agarose (Lonza) electrophoresis gel.

PCR-amplified fragments containing the V-Set domain were subcloned in a pSELECT-CHis-blasti (Invivogen) previous enzyme digestion with AgeI-HF and BamHI-HF (both from New England Biolabs) for 1 h at 37°C and ligated with T4 DNA ligase (New England Biolabs) for 3 h at RT (**Figure 37B**). PSELECT-CHis-blasti is an expression plasmid designed to generate polyhistidine (His) tagged proteins in order to facilitate their detection and/or purification. It also contains a blasticidin expression cassette, which permits to select those cells containing the plasmid. Plasmids clones were verified by DNA sequencing at the Genotyping and Sequencing Unit of UPV/EHU.

HEK-293T cells were transfected with the plasmids V-Set domain including or not the leader peptide using calcium phosphate, following manufacturer's protocol, and maintained with blasticidin selective antibiotic (Invivogen) (**Figure 37C**). Upon 24 h, cells and supernatant were collected and resuspended in lysis buffer (0.05% tween, 10 mM imidazole, 50 mM NaH_2PO_4, and 300 mM NaCl pH=8). Cells were lysed by sonication on ice (x6 cycles, 15 sec ON, 10 sec OFF 75W) and centrifuged to collect the supernatant. Supernatants were incubated in a pre-equilibrated Ni-Nta (nickel-nitrilotriacetic acid) agarose (Qiagen) for 3 h at 4°C to allow binding of the V-Set-His-tag protein to the matrix. Unbound protein was removed with washing buffer (0.05% tween 20, 20 mM imidazole, 50 mM NaH_2PO_4, and 300 mM NaCl pH=8). Protein was eluted with elution buffer (0.05% tween, 250 mM imidazole, 50 mM NaH_2PO_4, and 300 mM NaCl pH=8) in several steps. Cell pellet, flow-through, wash and eluted fractions

were collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

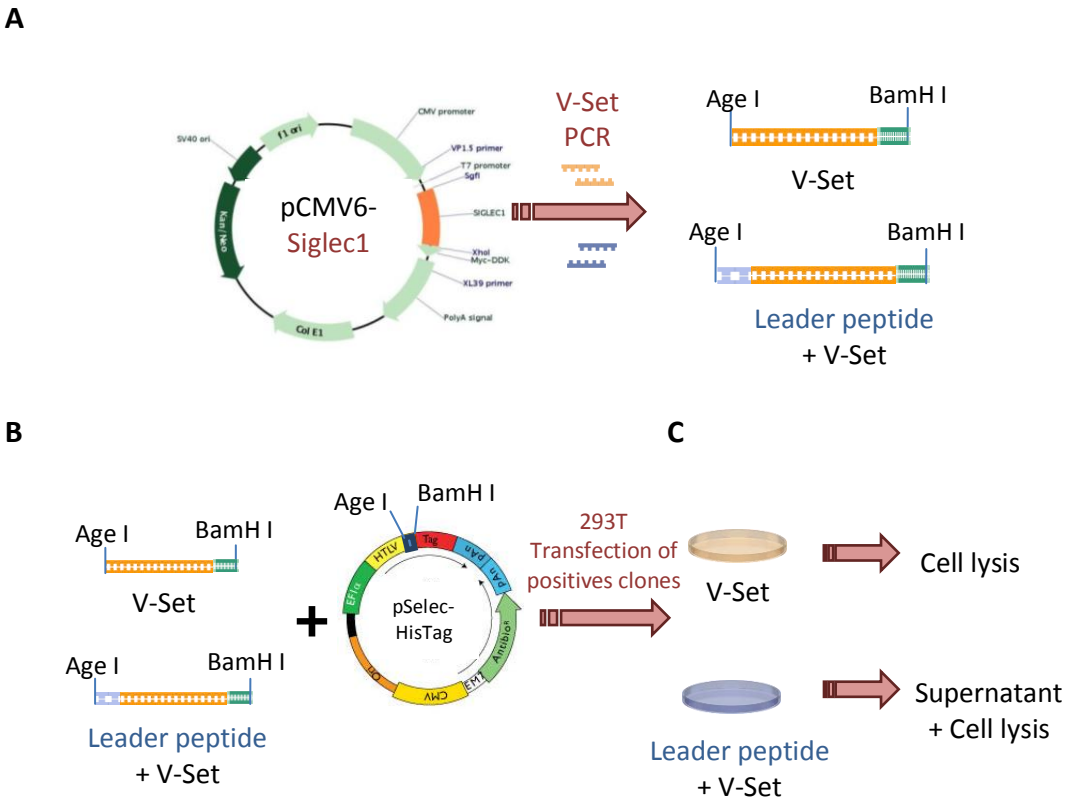


Figure 37. Design of V-Set domain immunogen. DNA molecular cloning and transfection scheme performed to produce recombinant V-Set protein. **A.** V-Set (amino acids 20 – 136) and V-Set plus leader peptide (1 – 136 amino acids) sequences were amplified by PCR from plasmid pCMV6 Siglec-1. **B.** PCR-amplified fragments were subcloned in a pSELECT-Chistag plasmid previous enzyme digestion with AgeI and BamHI. **C.** HEK-293T cells were transfected with the plasmids containing the V-Set domain. 24 h later, cells and supernatant were collected to purify the V-Set protein.

5.1. Quantitative Western blot of recombinant V-Set protein

To detect V-Set-His tag protein, samples were run on SDS-PAGE and transferred to a nitrocellulose membrane. The V-set-His tag protein content was determined by probing with an anti-histidine mAb (dilution 1:5000, Life Technologies), followed by an anti-mouse Ig G coupled with horseradish peroxidase (Cell Signaling Technology). The membrane was scanned with an Odyssey (LI-COR), and protein was quantified using Precision plus Protein Dual Color Standard (Bio-rad).

5.2. Silver staining of recombinant V-Set protein

Samples were run on SDS-PAGE and fixed in 12% acetic acid, 50% methanol, 38% distilled water (dH_20) and 500 µl of formaldehyde for 1 h. To remove acetic acid we performed three washes with 50% ethanol and dH_20. Gel was sensitized in 150 µl of 8.6% Na_2SO_4 in 100 ml dH_20 for 1 min. After three washes with dH_20, silver nitrate solution (0.2 g AgNO3, 200 ml H_20, 0.02% formaldehyde) was added for 20 min to allow deposition of metallic silver on macromolecules. After one washing, gel was developed in 3% Na_2CO_3, 0.05% formaldehyde in dH_20. When the staining was sufficient, gel was extensively washed with dH_20.

RESULTS

1. Small sialyllactose-like molecules do not block Siglec-1-mediated viral capture

To test whether candidate compounds could efficiently block Siglec-1 viral capture, a transient Siglec-1 expressing cell line was used to assess VLP binding by FACS. Cells were pre-incubated with increasing concentrations of the different compounds and then exposed to VLP. As a blocking control we used the natural ligand of Siglec-1, sialyllactose, which is able to inhibit VLP capture at high concentrations. Four out of 23 compounds tested (EST0065609, EST0065610, EST0065611 and EST0065865) affected cell viability at the highest concentration and were therefore discarded. Only three compounds (EST0065571, EST0065572 and EST0065573) diminished VLP capture, achieving the same blocking effect as sialyllactose (**Figure 38**). However, these compounds were only able to block viral capture at 10 mM (**Figure 38**). Thus, blocking Siglec-1 with small sialyllactose-like compounds requires high millimolar concentrations above the clinical accepted ranges (1nM to 1mM) [201].

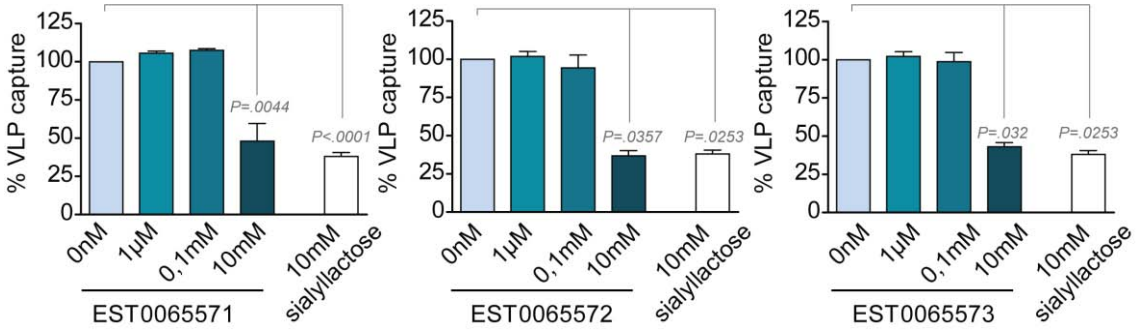


Figure 38. Small sialyllactose-like molecules do not block Siglec-1-mediated viral capture. Relative uptake of VLPs by Raji-Siglec-1 cells that had been pre-incubated with increasing concentrations (1 µM – 10 mM) of distinct compounds before VLP exposure for 2 h at 37°C. Values are normalized to the level of VLP captured by non-treated cells (set at 100%). One sample *t* test was used and *P* values are shown. Data show mean values and SEM from one experiment including duplicates.

2. Sialyllactose glycodendrons do not block Siglec-1-mediated viral capture

Because small sialyllactose-like compounds tested were not able to block Siglec-1, we next tested multivalent sialyllactose compounds that could increase Siglec-1 binding avidity, and thus, block HIV-1 capture and transmission.

When we performed a VLP capture assay using LPS mDCs, pre-treatment with a mAb α-Siglec-1 could efficiently block VLP capture **(Figure 39)**. However, pre-incubation with a high concentration of a sialic acid glycodendron had no impact on VLP capture, as neither pre-incubation with increasing concentrations of sialyllactose glycodendrons **(Figure 39)**.

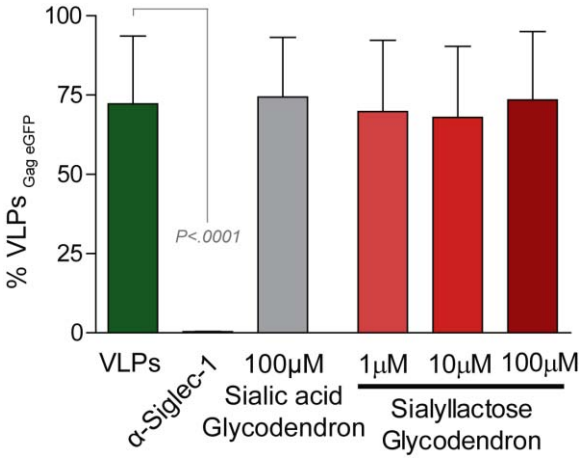


Figure 39. Sialyllactose glycodendron pre-treatment does not block VLP capture mDCs. Graph shows percentage of VLP positive mDCs. *P* values are calculated with a paired *t*-test. Data show mean values and SEM from one experiment including duplicates.

To assess if fluorescent sialyllactose glycodendrons specifically recognize Siglec-1, LPS mDCs were pre-treated with mAb α-Siglec-1 or matched isotype mAb and then pulsed with increasing concentrations of the sialyllactose glycodendron. mAb α-Siglec-1 could not block uptake of sialyllactose glycodendrons at any of the concentrations tested, indicating that glycodendron binding is independent of Siglec-1 recognition **(Figure 40)**. Thus, the multivalent siallylated compounds tested here are not enough to block Siglec-1 viral capture.

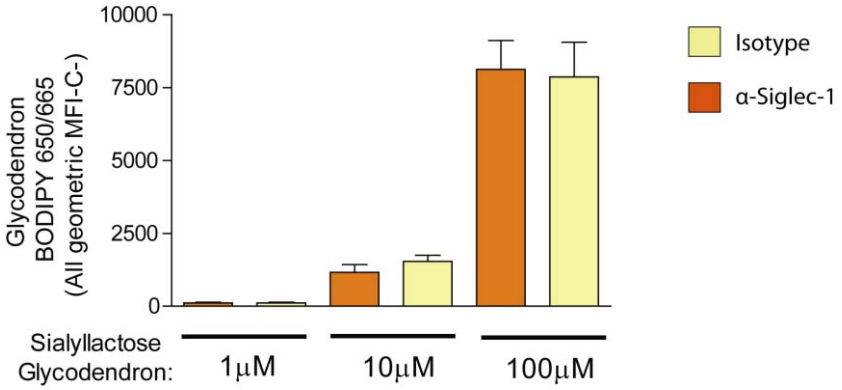


Figure 40. mAb α-Siglec-1 pre-treatment does not block fluorescent sialyllactose glycodendron capture by mDCs. Graph shows geometric mean fluorescence intensity (MFI) of sialyllactose glycodendron coupled to BODIPY 650/665.

3. Recognition of small immunogenic selected Siglec-1 peptides by antibodies from immunized mice is not sufficient to block Siglec-1

As an alternative approach to block Siglec-1, we also tried to develop a mAb against the V-set domain, the specific region for HIV-1 recognition. This amino-terminal V-Set domain interacts directly with sialylated ligands, with a preference for Neu5Ac in α2–3 and α2–6 linkages [143]. Immunogens corresponding to different regions of the Siglec-1 V-Set domain were used to vaccinate three groups of 5 female mice BALB/c for each peptide. After careful peptide design (**Figure 35**), we synthesized the following peptides: Sig20 (amino acids 20-36); Sig113 (amino acids 113-127); and Sig122 (amino acids 122-134). Following immunization (**Figure 36**), we performed an ELISA in order to detect the presence of Abs against the synthetic linear peptides in the mouse sera. Sera from mouse immunized with a non-related peptide was used as negative control.

Sera from the three immunizations presented a good specific recognition of linear synthetic peptides (**Figure 41**). Although signal was higher from Sig20 immune sera, all sera showed a low antibody titer. Thus, mice immunized with small synthetic linear Siglec-1 peptides produced antibodies that specifically recognized their own peptide used for immunization, but with a low titer.

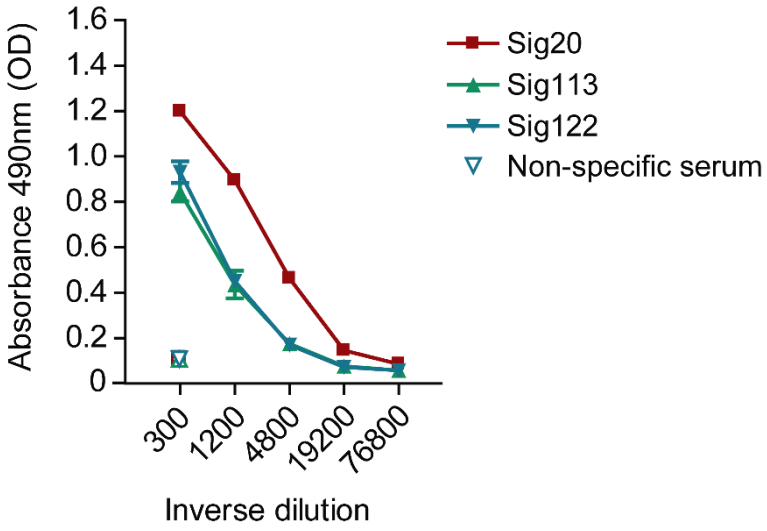


Figure 41. Mice immunized with small synthetic linear Siglec-1 peptides produce antibodies that specifically recognize the immunogenic peptides. ELISA of a representative serum from each immunization group with Sig20, Sig113 and Sig122 is shown. Plates coated with each antigen were probed with serial dilutions of sera (range from 1/300 to 1/76800) from each type of immunization. Clear symbols show the recognition of the Sig20, Sig113, and Sig122 peptides by the serum of a control peptide immunization.

To determine if serum from immunized mice could block Siglec-1 viral recognition, we performed a VLP capture assay. Raji Siglec-1 were pre-incubated with serial dilutions of sera before VLP exposure. As a blocking control, we used the mAb α-Siglec-1. As a negative control, we used the sera from a non-related mice immunization. Although VLP capture could be reduced with the mAb α-Siglec-1, none of the sera from mice immunized with the three peptides could block VLP capture (**Figure 42**).

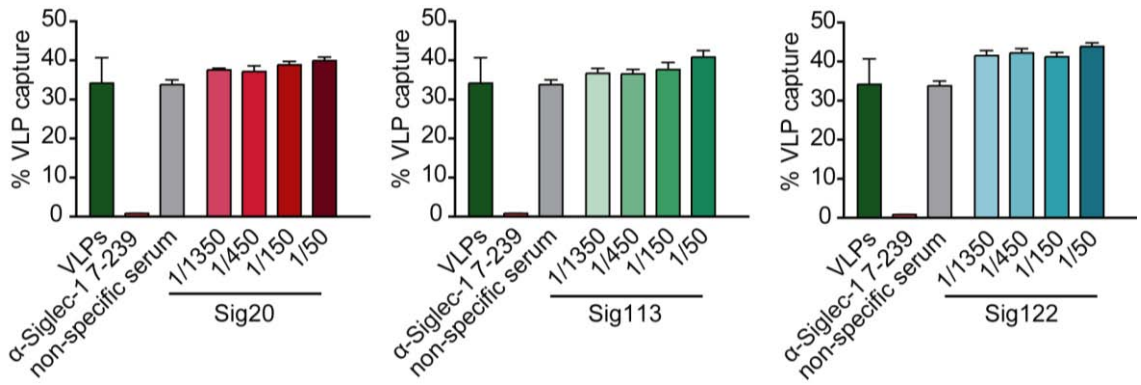


Figure 42. Sera from mice immunized with small synthetic Siglec-1 peptides are not able to block VLP capture. Capture of VLP by Raji-Siglec-1 cells that had been pre-incubated with mAb α-Siglec-1 7-239, non-specific serum or serial dilutions of the indicated sera from immunized mice. Graph shows mean values and SEM from one experiment where each serum was tested independently and by duplicate.

To confirm whether the lack of Siglec-1 blocking activity of sera is due to a non-recognition of Siglec-1 receptor, Raji Siglec-1 cell line was stained with unlabeled mAbs α-Siglec-1 7-239, 7D2 or sera from immunized mice. A labeled antibody against mouse IgG was used as a secondary antibody to reveal primary antibody binding by FACS. While secondary mAb detected the binding of control mAbs α-Siglec-1 7-239 and 7D2 to Siglec-1, it could not detect the binding of any of the sera, indicating the lack of Siglec-1 recognition (**Figure 43**).

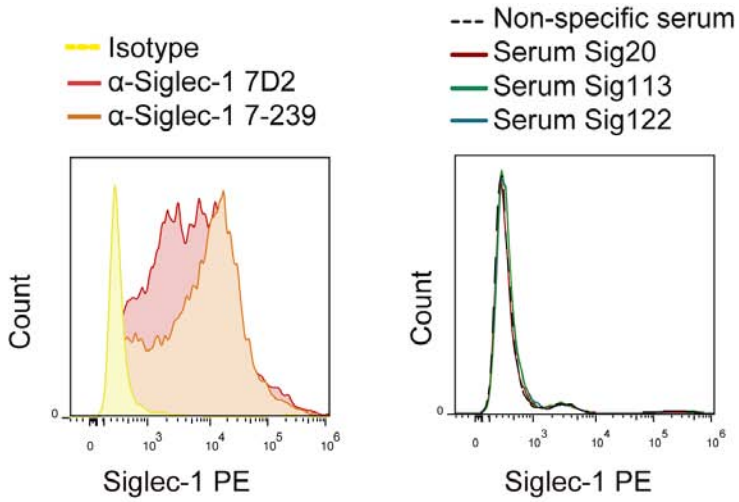


Figure 43. Sera from mice immunized with small synthetic Siglec-1 peptides do not recognize Siglec-1 receptor. Representative Siglec-1 staining of Raji Siglec-1 cells labelled with commercial mAbs α-Siglec-1 7-239 and 7D2, non-specific sera or the indicated sera from immunized mice. Cells were stained with a PE secondary Ab.

We next asked if the available commercial blocking mAbs α-Siglec-1 7-239 and 7D2 (Hartnell 2001)[72] could recognize lineal synthetic peptides Sig20, Sig113 and Sig122 used in the immunizations. When we performed an ELISA, none of the commercial blocking mAbs were able to recognize any of the selected synthetic peptides (**Figure 44**).

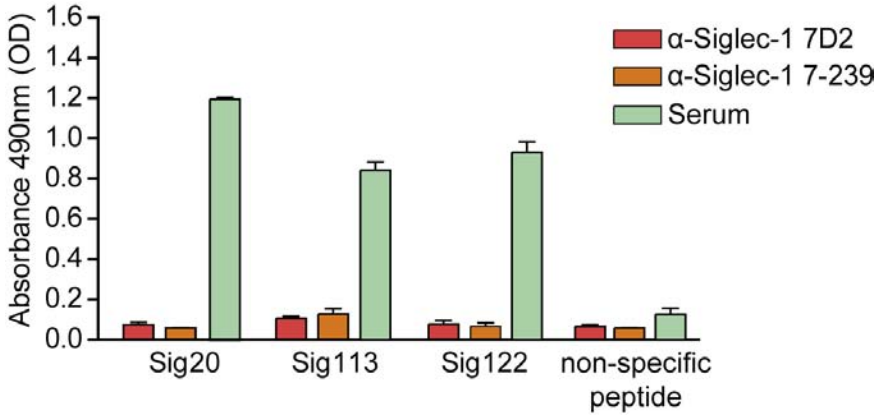


Figure 44. Commercial Siglec-1 mAbs do not recognize linear synthetic selected Siglec-1 peptides from V-Set domain. Recognition of Siglec-1 peptides Sig20, Sig113, Sig122 or non-specific peptide by the commercial mAbs α-Siglec-1 detected with an ELISA. Sera from immunized mice with the selected epitopes were used as positive controls. Graph shows mean values and SEM from one experiment.

Overall, these data demonstrate that Abs present in the sera of mice immunized with small V-Set synthetic peptides Sig20, Sig113 and Sig122 were not able to recognize Siglec-1, and consequently, cannot block viral uptake.

Since small immunogenic peptides from the V-Set domain did not induce the production of mAbs recognizing the three-dimensional structure of the Siglec-1 receptor expressed on the cellular surface, we next attempted to use the complete V-set domain of Siglec-1 as an immunogen. The rational of this approach is that if we maintain protein structure and glycosylation in the whole region implicated in HIV-1 recognition, we could increase the probability of producing anti-Siglec-1 blocking Abs.

Based in these results, we aim to produce a Siglec-1 recombinant protein containing only the V-set domain. By this means, the protein produced will maintain the conformational structure resembling more to the original immunogen and will allow us to purify large protein quantities required for immunization. Thus, we modified the Siglec-1 plasmid in two different ways: one containing the V-Set domain only and the other one containing the Siglec-1 leader peptide as well (**Figure 45A)**. This leader

peptide was introduced to produce a V-Set protein that could be secreted to the extracellular media, facilitating protein production and purification.

Plasmid modification was verified by DNA sequencing, and confirmed that both plasmids had incorporated a linker motif composed of serial Glycine-Serine and a Histidine tag at the C-terminal domain (**Figure 45B**). This linker is a flexible spacer that is important for connecting different domains in a single protein without interfering with the function of each domain.

Following transfection of HEK293T cells, V-Set proteins were purified with Ni-NTA (nickel-nitrilotriacetic acid) agarose and quantified by Western Blot and Silver Staining. Cells transfected with the V-Set plasmid expressed our protein of interest as revealed by Western Blot (**Figure 45C**). However, a high level of unspecific proteins was found as well, as analyzed by silver staining (**Figure 45D**). Moreover, cells transfected with the plasmid encoding for the V-Set domain plus the leader peptide did not secrete it to the media despite correctly expressing our protein in the cell lysate (**Figure 45E**).

Thus, although DNA molecular cloning and transfection assays resulted in successful production of Siglec-1 V-Set domain, protein purification procedures need further optimization to obtain enough immunogen with high purity.

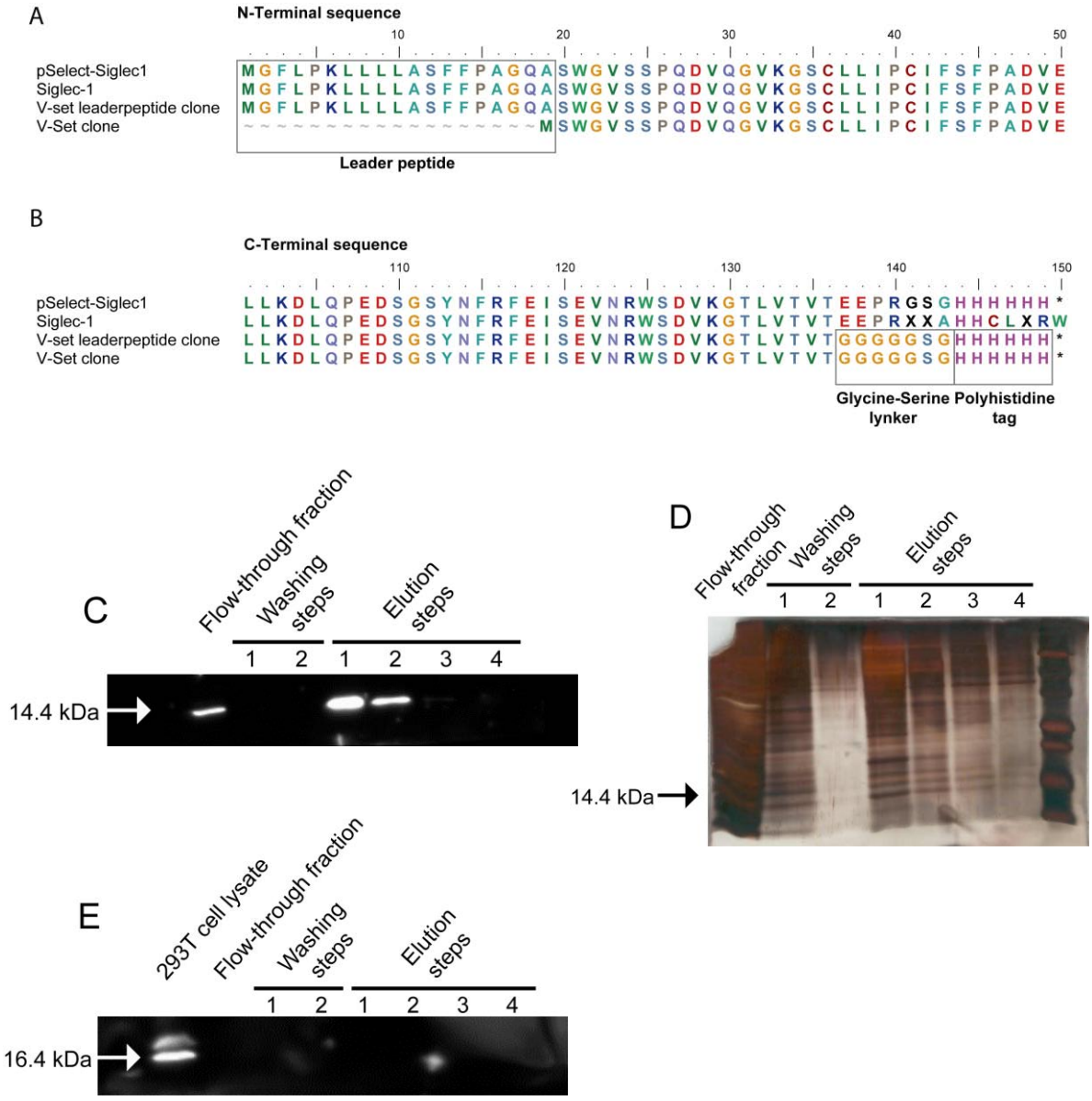


Figure 45. A. N-terminal alignment of amino acid sequences of: Siglec-1 plasmid, Siglec-1 reference sequence, and the designed plasmids with or without the leader peptide (outlined in a box). **B.** C-terminal alignment of amino acid sequences of: Siglec-1 plasmid, Siglec-1 reference sequence, and the designed plasmids. Glycine-Serine linker and polyhistidine tags are outlined in a box. **C.** Western Blot analysis of lysed HEK293T cell transfected with the V-Set plasmid. **D.** Silver staining of lysed HEK293T cell transfected with the V-Set plasmid **E.** Western Blot analysis of lysed HEK293T cell transfected with the V-Set plasmid plus the leader peptide and their supernatant.

In summary, our future goal is to produce compounds to block Siglec-1 recognition of HIV-1 to diminish viral dissemination and reduce HIV-1 pathogenesis. Our different strategies have not yielded positive results yet, but we continue actively working to ameliorate these systems to identify potential small blocking inhibitors and produce effective mAbs that could become a valuable tool in future therapeutic strategies.

Chapter 7

DISCUSSION

1. Immune activation is a driver of HIV-1 pathogenesis and is fueled by Siglec-1

HIV-1 infection is characterized by a marked increase in immune activation, which affects both the adaptive and the innate immune systems. The drivers for HIV-1 immune activation include i) the direct effects of HIV-1 as a ligand for endosomal TLR7 and TLR9, leading to over-production of IFNα by IFN-producing cells such as pDCs [202], and; ii) the increased microbial translocation, where the structural bacterial component LPS acts as a potent activator of cell surface TLR4, leading to the production of pro-inflammatory cytokines such as IL-6, TNFα and type I IFN by myeloid cells [203]. These immune activation factors are present since the very early stages of HIV-1 infection and continue to play a role over the chronic phases. IFNα is an antiviral cytokine that is able to block cell-free HIV-1 infection in cell culture models via the up-regulation of IFN-stimulated genes that encode viral restriction factors [204–208]. However, neither IFNα secretion by pDCs exposed to HIV-1 during acute infection, nor exogenous administration of IFNα to HIV-1-infected patients has been able to fully suppress viremia [150, 209, 210].

In this thesis, we propose that persistent chronic immune inflammation driven by IFNα present during HIV-1 infection induces Siglec-1 expression on myeloid cells, a key factor that promotes viral *trans*-infection, which fuels immune inflammation, and consequently, contributes to HIV-1 disease progression. We have observed that even low concentrations of the pro-inflammatory cytokine IFNα detected in HIV-1-infected patients [211] induce Siglec-1 on DCs (**Figure 16**). Thus, during HIV-1 infection, which is characterized by the persistent release of IFNα, Siglec-1 receptor could be augmented on myeloid cells and contribute to disease progression. Furthermore, we have confirmed that Siglec-1 induction results from HIV-1 recognition by pDCs (**Figure 17**), which release of soluble factors upon viral recognition, such as IFNα [143, 144] (**Figure 46**). Although pDCs decrease in blood during primary and chronic HIV-1 infection [212, 213], high levels of IFNα are detected in plasma during both acute and chronic HIV-1 infection [150]. pDCs circulating in blood have the ability to migrate to sites of inflammation and to secondary lymphoid tissues, where they contribute to the

inflammatory milieu [161, 187]. Upon HIV-1 infection, migrating pDCs present a partial matured phenotype, which concurs with a persistent IFNα stimulation [153]. Thus, the continuous production of IFNα could partially accelerate HIV-1 pathogenesis by increasing Siglec-1-mediated viral spread, and vice versa.

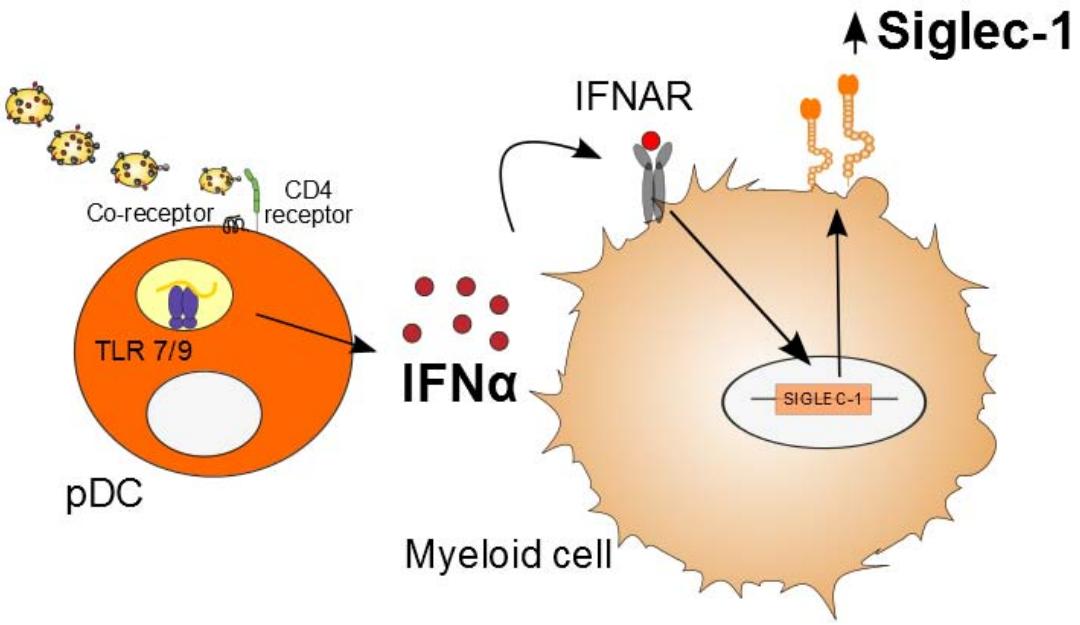


Figure 46. **IFNα released by HIV-1-exposed pDCs induces Siglec-1 expression on myeloid cells.** Pathogen-associated ssRNA and CpG DNA recognition through endosomal TLR7 and TLR9 on pDCs respectively, triggers IFNα secretion that induces Siglec-1 expression via signaling of type I IFN receptor.

We have also shown that Siglec-1 was up-regulated upon IFNα treatment, not only in DCs but also in other myeloid cells such as macrophages and monocytes (**Figure 18**). Siglec-1 was first described as a constitutive receptor expressed in macrophages [143]; however, IFNα treatment also up-regulates Siglec-1 expression on macrophages [149]. Accordingly to our results, it has been demonstrated that exposition to exogenous IFNα up-regulates Siglec-1 expression on monocytes [144] and DCs [136]. Indeed, the *in vivo* effect of exogenous IFNα on Siglec-1 induction on monocytes was recently confirmed in a clinical study from our group, where short-term treatment with IFNα plus Ribavirin in HIV-1-infected patients resulted in a significant increase in Siglec-1 expression on monocytes at day 28 post-treatment [214]. Furthermore, Siglec-1 has also been detected on monocytes and macrophages from patients suffering viral infections or autoimmune diseases, which are characterized by a persistent production

of type I IFN, such as systemic lupus erythematosus [147] and systemic sclerosis [144]. Therefore, our results indicate that release of IFNα via HIV-1 activation of pDCs induces Siglec-1 in an otherwise antiviral environment established by IFNα.

2. HIV-1 exploits myeloid cells for viral spread via Siglec-1 receptor

We next asked whether Siglec-1 receptor could mediate HIV-1 uptake and *trans*-infection in activated myeloid cells. Several studies have characterized Siglec-1 on activated DCs as an IFN-inducible receptor that recognizes HIV-1 membrane gangliosides and mediates viral uptake and *trans*-infection to $CD4^+$ T cells, contributing to viral spread during HIV-1 infection [135, 136]. Regarding the role of Siglec-1 on monocytes and macrophages, we have shown that Siglec-1 mediates HIV-1 uptake and *trans*-infection not only in DCs, but also in macrophages and monocytes (**Figure 21 and 24**). Some studies have determined that Siglec-1 on monocytes and macrophages recognizes sialyllactose motifs but from the viral envelope glycoprotein [106, 215]. However, by using VLPs that lack the envelope glycoprotein, here we have provided evidences that increased viral uptake in IFNα-activated monocytes and macrophages takes place via recognition of viral membrane gangliosides (**Figure 22**). Furthermore, specific blockade of Siglec-1 receptor resulted in a significant decrease in HIV-1 uptake and *trans*-infection in all myeloid cells (**Figures 21 and 24**).

However, a striking distinct viral uptake and *trans*-infection capacity was observed among distinct myeloid cells. While DCs and monocytes presented similar uptake and *trans*-infection capacity, macrophages displayed a much lower viral transfer capacity (**Figure 21 and 24**). Macrophages possess scavenger functions, and engulf and degrade invading pathogens through lysosomal degradation pathways [216]. By blocking lysosome activity we have demonstrated that captured virions in macrophages presented faster viral degradation kinetics, and consequently, macrophages presented a reduced viral *trans*-infection (**Figure 21**). Yet, several studies indicate that Siglec-1 expression in macrophages might facilitate productive HIV-1 *cis*-infection [215], what could fuel HIV-1 pathogenesis through direct macrophage infection despite their limited *trans*-infection capacity.

To elucidate HIV-1 trafficking differences in macrophages compared to other myeloid cells, we investigated viral uptake using microscopy. Confocal images revealed that HIV-1 was internalized into Siglec-1$^+$ compartments in DCs and monocytes, whereas HIV-1 presented a random punctuate distribution on macrophages (**Figure 23**). The observed viral distribution supports the differences detected in viral uptake and *trans*-infection between DCs/monocytes and macrophages (**Figure 47**). Thus, faster viral degradation of captured virions in macrophages did not allow us to detect well-defined viral compartments. In contrast, several studies have identified viral intracellular compartments in HIV-1-infected macrophages, which concentrate newly produced virions in plasma membrane-derived intracellular invaginations that are CD81$^+$, CD82$^+$ and CD9$^+$ [113–115]. In our viral capture assays we never observed these viral compartments in macrophages because they were not productively infected. Thus, in our studies we focused on viruses accumulated by endocytosis, but not on newly formed budding virions that would require a different tropism to infect macrophages and an observation performed at later time points (more than 24h) to allow for new viral particle production.

In monocytes and DCs, the formation of a viral compartment might allow HIV-1 hiding from antiretroviral treatment and immune control. Although viruses can be retained at this compartment for up to four to five days, they are however progressively degraded [108]. This structure is accessible to surface probes [109] but inefficiently accessed by neutralizing antibodies [108, 217], indicating that this sac-like compartment precludes antibody neutralization. Thus, the formation of Siglec-1$^+$ viral compartments on DCs and monocytes could favor viral accumulation, evasion and transmission to uninfected CD4$^+$ T cells, while in macrophages HIV-1 is quickly degraded and hence poorly transmitted to target cells (**Figure 47**). Furthermore, based in our results, HIV-1 accumulation and posterior polarization of virions towards a cell-to-cell infectious synapse is able to overcome the antiviral effect of IFNα *in vitro* (**Figure 25**). IFNα has been described as a cytokine that restricts HIV-1 replication in CD4$^+$ T cells [204–208, 218]. However, cell-to-cell HIV-1 transfer is less sensitive to IFNα than infection by cell-free virions. Thus, viral transfer between CD4$^+$ T cells is partly inhibited by type I IFN [219]. By performing *trans*-infection assays in the presence of IFNα, we have observed

a partial reduction of viral transmission via Siglec-1 (**Figure 25**). Furthermore, Siglec-1-mediated viral transmission by DCs was higher compared to viral transmission between $CD4^+$ T cells (**Figure 25**). Thereby, in an inflammatory milieu, where there is a progressive accumulation of IFNα, Siglec-1-mediated viral transmission might allow a more effective viral replication than those infectious mechanisms independent of Siglec-1.

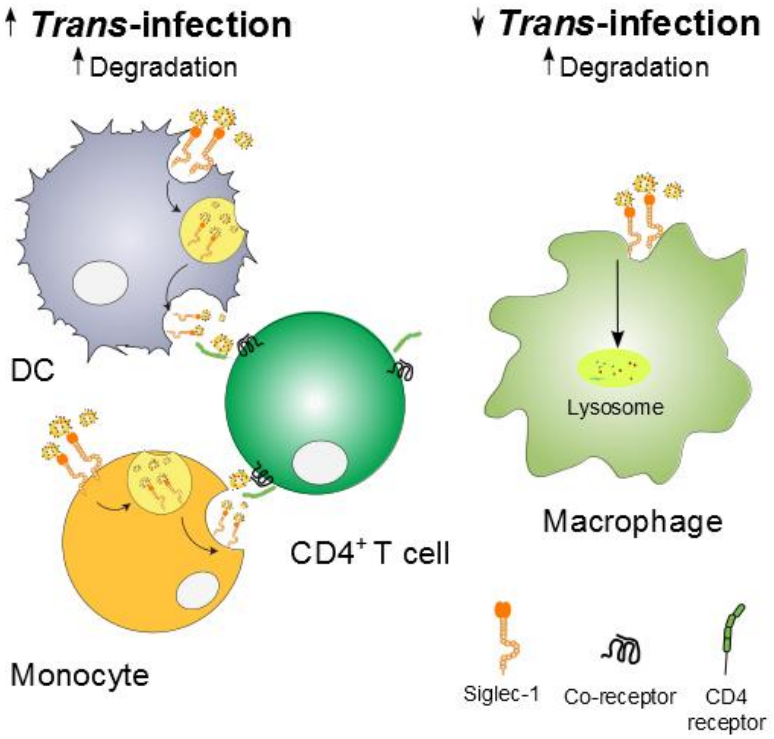


Figure 47. Siglec-1 mediates HIV-1 uptake into a storage compartment and enhances HIV-1 *trans*-infection especially in IFNα-treated monocytes and DCs. Siglec-1 expressed on monocytes and DCs is able to store and transfer HIV-1 to uninfected target $CD4^+$ T cells. However, Siglec-1 on macrophages internalizes HIV-1, which is subsequently degraded reducing HIV-1 *trans*-infection to $CD4^+$ T cells.

3. HIV-induced immune activation regulates Siglec-1 expression on monocytes from HIV-1-infected patients

Since Siglec-1 expressed on IFNα-activated myeloid cells mediated viral capture and transmission to bystander $CD4^+$ T cells *in vitro* **(Figures 21 and 24)**, we next asked whether Siglec-1 could be expressed on myeloid cells *in vivo* and contribute to HIV-1 dissemination throughout infection. To determine whether immune activating factors

present in the plasma of HIV-1 infected individuals could induce Siglec-1 on myeloid cells, we assessed Siglec-1 expression on circulating monocytes directly isolated from untreated HIV-1-infected patients with high viremia. We found that Siglec-1 expression was up-regulated on monocytes from untreated HIV-1-infected individuals (**Figure 26**) and was positively correlated with viral load and inversely correlated with $CD4^+$ T cell count (**Figure 28**). Furthermore, we observed a significant down-regulation of Siglec-1 expression upon initiation of HAART (**Figure 26**). Accordingly, a study of Siglec-1 expression during different stages of HIV-1 infection showed that Siglec-1 is induced on monocytes early after HIV-1 infection and maintained during disease progression in untreated HIV-infected patients [179]. These results were recapitulated in a longitudinal study of SIV infection in the pathogenic model of *Rhesus macaques* [220], where monocytes up-regulated Siglec-1 during the acute phase and, after a slow decrease following the peak of viremia, its expression continued to increase during progression to AIDS. As we have shown for HIV-1-infected patients, SIV-infected *Rhesus macaques* also diminished Siglec-1 expression on monocytes as a result of successful antiretroviral treatment [220]. Moreover, Siglec-1 expression on monocytes abruptly increased upon experimental depletion of cytotoxic $CD8^+$ T lymphocytes, along with the concomitant increase in SIV plasma viremia [220]. Thus, Siglec-1 expression is associated with plasma viral load throughout the course of HIV/SIV infection in pathogenic hosts. Importantly, though, up-regulation of Siglec-1 is not observed in non-pathogenic species, such as *Sooty Mangabyes* [221]. These findings are consistent with the lack of immune activation in *Sooty Mangabyes* [154]. Taken together, our results along with those reported elsewhere suggest that unsuppressed viremia might induce the release of inflammatory signals that up-regulate Siglec-1 expression on circulating monocytes. Thus, Siglec-1 expression on monocytes could be used as a biomarker of virological failure and chronic immune activation.

We have also observed that increased Siglec-1 expression on circulating monocytes from untreated HIV-1-infected patients was positively correlated with HIV-1 uptake and *trans*-infection capacity *ex vivo* (**Figure 26**). This concurs with a previous study highlighting the role of Siglec-1 in HIV-1 *trans*-infection in a monocytic cell line model [106]. However, here we have demonstrated that primary monocytes isolated from

HIV-1-infected patients mediate *trans*-infection via Siglec-1. In sharp contrast, when HAART suppressed viremia, Siglec-1 expression on monocytes was diminished, and consequently, viral uptake and *trans*-infection also decreased (**Figure 26**). Nevertheless, residual viral *trans*-infection present during antiretroviral treatment could be an important source of persistent viral replication fueling residual inflammation. Indeed, residual viral replication under HAART partially drives immune activation [222] as seen with intensification treatments with raltegravir that were able to reduce the extent of immune activation by further suppressing residual viral replication, at least in some patients [223].

Residual or active viral replication triggers the release of type I IFN that is detected in the plasma of HIV-1 infected individuals [150]. We have observed that plasma of untreated HIV-1-infected patients augmented Siglec-1 expression to a higher extent than plasma from HAART treated patients (**Figure 27**). Remarkably, Siglec-1 induction could be blocked by abrogating type I IFN receptor activity (**Figure 27**). This does not necessarily mean that type I IFN accumulated in plasma is directly inducing Siglec-1 expression on circulating monocytes, since LPS from microbial translocation also accumulates in the plasma of HIV-1-infected individuals [150] and induces autocrine type I IFN production via TLR4/CD14 signaling [188] (**Figure 48**). Plasma levels of LPS are increased in chronically HIV-1-infected patients and SIV-infected macaques, but are reduced after effective HAART [150, 159, 160]. Furthermore, LPS levels correlate with IFNα levels in plasma, the expression of IFN-stimulated genes, and the induction of biomarkers of inflammation and disease progression [150]. Future experiments specifically blocking either LPS or type I IFN pathways would clarify which is the major stimulus present in the plasma of HIV-1-infected patients that triggers Siglec-1 expression on myeloid cells.

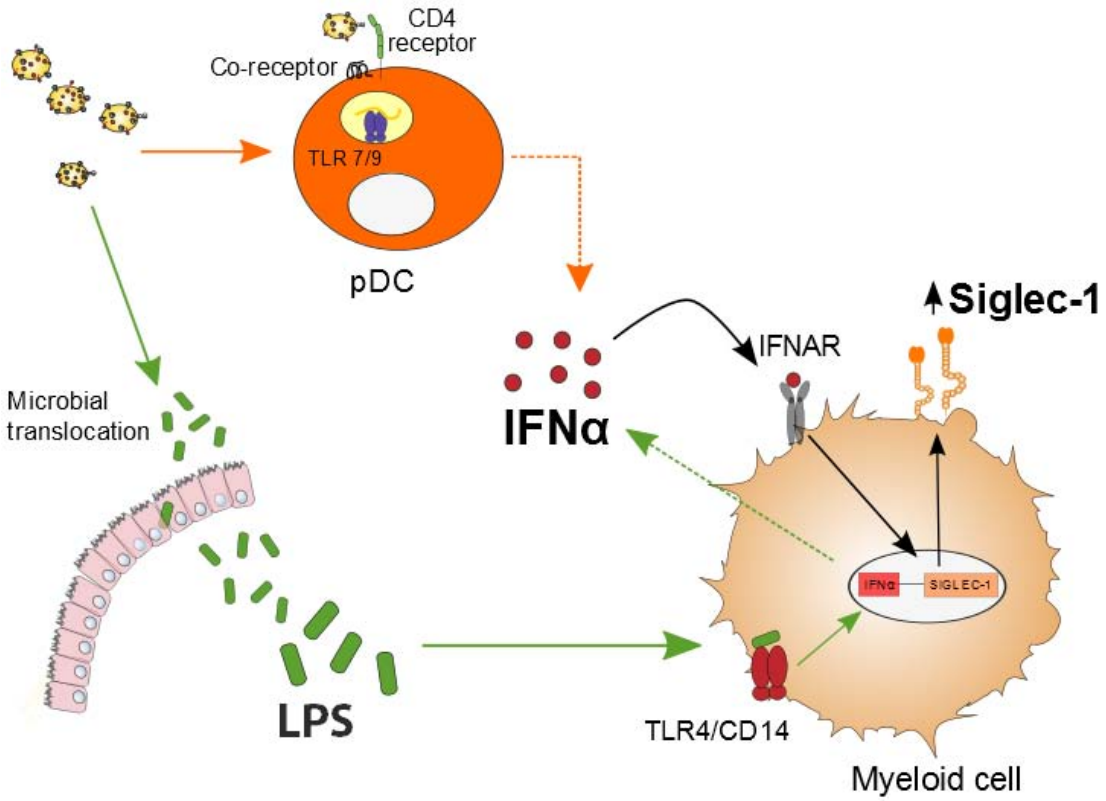


Figure 48. **IFNα induces Siglec-1 expression on myeloid cells through a direct or an indirect secretion pathway**. Indirect pathway (green arrows): HIV-1 triggers microbial translocation, which increases LPS levels in plasma that will induce autocrine IFNα secretion through TLR4/CD14 signalling on myeloid cells and induce Siglec-1 expression. Direct pathway (orange arrows): HIV-1 infection triggers endosomic TLR7/9 recognition by pDCs that results in IFNα release and subsequent Siglec-1 induction.

Regardless of the stimuli present in the plasma of HIV-1 infected individuals, Siglec-1 expression on circulating monocytes induced by uncontrolled viremia mediates HIV-1 uptake and has the potential to *trans*-infect uninfected $CD4^+$ T cells (**Figure 26**). *In vivo*, however, it is unlikely that viral *trans*-infection could occur in cells circulating in the peripheral blood: cell-to-cell encounters within bloodstream are transient and less frequent than in tissues, where monocytes rapidly migrate upon activation [224]. Once circulating monocytes harboring HIV-1 migrate to tissues, viral spread to uninfected target cells could be enhanced (**Figure 49**), although further studies in animal models could shed light into this process. Indeed, the establishment of HIV-1-associated neurocognitive disorders is largely attributed to monocyte transmigration into the central nervous system, particularly that of a $CD14^+CD16^+$ monocyte population [224, 225]. Therefore, the presence of immune activating factors in the plasma of HIV-1-

infected patients with high viremia could up-regulate Siglec-1 on circulating monocytes facilitating HIV-1 uptake, and subsequent monocyte activation and migration to tissues where cell-to-cell viral transmission could occur.

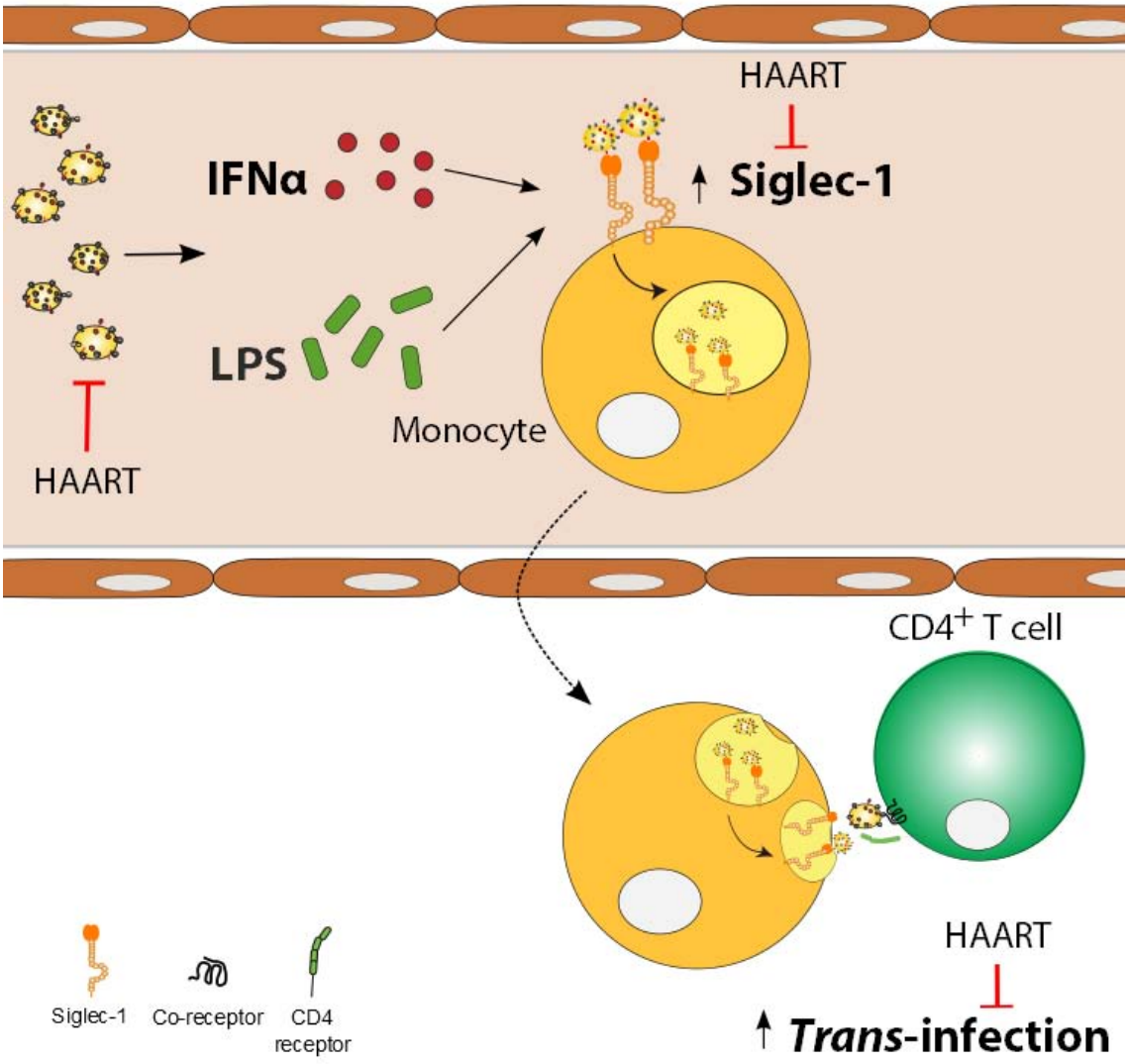


Figure 49. Siglec-1 mediates *trans*-infection on monocytes from HIV-1-infected patients. High viremia triggers increased IFNα and LPS levels on plasma, which leads to an up-regulation of Siglec-1 expression on circulating monocytes that facilitates viral capture. Once monocytes harboring HIV-1 are activated, they transmigrate into tissues where *trans*-infection could occur. Introduction of HAART reduces viremia, which concomitantly reduces immune activation, Siglec-1 expression and HIV-1 *trans*-infection.

4. Siglec-1$^+$ myeloid cells contribute to HIV-1 spread on activated lymphoid tissues

Lymphoid tissues are the perfect scenarios to fuel novel infections via Siglec-1 expressed on myeloid cells, since these tissues are major sites of HIV-1 replication [196], where plasmacytoid and myeloid cells accumulate during the course of HIV-1 infection [161, 162, 226] and IFNα is preferentially accumulated [166, 180].

The migration and accumulation of pDCs [161, 162], and accumulation of type I IFN in lymphoid tissues [166, 227] could induce Siglec-1 expression on myeloid cells during chronic HIV-1 infection (**Figure 50**). Thus, we aimed to determine whether this inflammatory milieu present at the lymphoid tissue during chronic HIV-1 infection [166] could induce Siglec-1 expression *in vivo*. Since obtaining lymphoid tissues from untreated HIV-1-infected patients to perform immunohistochemistry is difficult, we extrapolated the analysis of lymphoid tissues from HIV-, HBV- and HCV-negative subjects that were characterized by high inflammation, and compared them to non-inflamed tissues (**Figure 30**). Immunohistochemistry analysis revealed that Siglec-1 expression was increased in inflamed lymphoid tissues and showed a clear association with the degree of immune activation (**Figure 30**). Moreover, we also found Siglec-1 expression in lymphoid tissues from an HIV/HCV co-infected patient (**Figure 30**). Siglec-1$^+$ cells of the HIV-1-infected individual were located in perivascular, perifollicular and subcapsular regions of the lymphoid tissue that are enriched in CD4$^+$ T cells. This disposition facilitates the interaction between Siglec-1$^+$ and bystander CD4$^+$ T cells, favoring viral *trans*-infection. Accordingly, human Siglec-1$^+$ cells have been found forming a rim under the subcapsular sinus and lining around arteriolar vessels in lymph nodes and spleen in HIV-1-negative individuals [228]. Furthermore, Siglec-1$^+$ cells locate close to systemic circulation [229], which could facilitate contact with circulating cell-free virus enhancing HIV-1 capture.

Although antiretroviral treatment reduces viremia and immune activation in HIV-1-infected patients, Siglec-1 expression differences before and after initiation of antiretroviral treatment were not observed in lymphoid tissues removed from this HIV/HCV co-infected patient (**Figure 30**). Lack of differences in Siglec-1 expression

could be due to the untreated HCV infection that triggers immune activation, which in turn, could lead to the up-regulation of Siglec-1 receptor even in the presence of HAART. Indeed, different studies found increased levels of immune activation in HCV/HIV-1 co-infected subjects despite effective HAART as compared to HIV-1 mono-infected subjects [230, 231]. Alternatively, limited penetration of HAART within the lymphoid tissues [194] could lead to residual viral replication resulting in an insufficient reduction of the immune activation [223, 232].

To examine whether Siglec-1^+ cells from lymphoid tissues were able to mediate viral capture and transmission, we performed functional assays with Siglec-1^+ tonsillar cells directly isolated from fresh tissue. Given that obtaining fresh lymphoid tissue from untreated HIV-1-infected individuals is complicated, we used instead fresh tonsils from HIV-1-negative individuals that were subsequently exposed *ex vivo* to IFNα as a model to mimic chronic HIV-1 infection (**Figure 29**). As expected, Siglec-1 expression was augmented in tonsillar cells upon IFNα treatment. To determine which cell type was the Siglec-1^+ population in IFNα-treated tonsils, we isolated these cells that only represented the 0.006% of all mononuclear tonsillar cells (**Figure 29**). Comparative transcriptome analyses showed that Siglec-1^+ tonsillar cells clustered separately from a lymphoid lineage but closer to DCs, monocytes and macrophages, suggesting their intimal relationship to the myeloid lineage (**Figure 31**). However, Siglec-1^+ tonsillar cells represented a separated cluster from DCs, monocytes and macrophages. A more detailed analysis of different genes related to the myeloid cell lineage confirmed that Siglec-1^+ tonsillar cells have a unique myeloid antigen-presenting cell profile (**Figure 31**). The presence of genes such as CCR7, CCL19, RANTES, MIP1α and MIP1β, involved in homing of myeloid cells confirmed the tonsillar origin of Siglec-1^+ cells [233]. When comparing transcriptome analyses of Siglec-1^+ tonsillar cells with distinct cell lineage markers compiled in the literature [14], Siglec-1^+ tonsillar cells shared the common dendritic cell precursor-derived DC lineage markers IRF8 and ftl3. Furthermore, they have IRF4, BATF3 and ZBTB46 lineage markers of precursor conventional DCs (pre-cDC) and they do not present the precursor pDC marker E2-2 (pre-pDC). However, when trying to classify Siglec-1^+ tonsillar cells with classical cell surface markers (BDCA2, CD11b, CD11c, CD141, etc.) transcriptomic analyses resulted in confounding results.

One possible explanation to these observations is that different myeloid cell subpopulations could express Siglec-1 within the tonsil. Distinguishing DC, monocyte or macrophage populations within the myeloid lineage is complicated, since these cells share multiple cell surface markers and antigen-presenting functions [234]. As an example, Siglec-1 has always been used as a macrophage marker expressed on macrophages in mice [235]; however, some studies found that some of these Siglec-1$^+$ cells express the restricted DC cell surface marker ZBTB46 [236–238]. Furthermore, immune activation alters the phenotype and function of the cells, what complicates to a higher extent the discrimination between distinct myeloid cells under inflammatory conditions. It has been recently suggested that when categorizing myeloid cells into monocytes, DCs or macrophages, we should take into account not only cell surface markers and functional characteristics, but also the ontogeny and activation status [29].

Functional assays performed here with tonsillar myeloid cells activated with IFNα have identified Siglec-1 as a key receptor involved in viral capture and transmission (**Figures 32 and 33**). Accordingly, myeloid cells migrate and accumulate into lymphoid tissues during HIV-1 infection [187]. During chronic infection, HIV-1 is already replicating in secondary lymphoid tissues and has access to susceptible target cells accumulated in a close spatial proximity, favoring cell-to-cell viral spread [161, 162, 239]. Indeed, mathematical models predict that only ~ 10% of the infections in the lymphoid tissue are originated by cell-free virions, while infected cells transmit the remaining ~ 90% [94]. Other studies, however, calculate that infected cells transmit approximately 60% of the novel infections [240]. We have found that Siglec-1$^+$ tonsillar cells are able to store HIV-1 in an intracellular compartment (**Figure 32**) confirming that the observed viral accumulation *in vitro* (**Figure 23**) can also originate in lymphoid tissues *ex vivo.* Hence, peripheral migrating DCs and resident activated DCs could uptake HIV-1 in Siglec-1$^+$ compartments and transmit the virus *in trans* to CD4$^+$ T cells in these sites (**Figure 50**). Although Siglec-1$^+$ tonsil cells represent a minor cell population, we believe that Siglec-1-mediated *trans*-infection is able to amplify CD4$^+$ T cell infection, even with small viral inputs found in lymphoid tissues in HAART treated HIV-1-infected patients [241]. Thus, persistent Siglec-1-mediated viral spread could play a role in the

establishment and maintenance of viral reservoirs in lymphoid tissues from chronic HIV-1-infected patients. Our data demonstrate the importance of Siglec-1-mediated *trans*-infection by human tonsillar myeloid cells in lymphoid tissues. Overall, we provide several lines of evidence suggesting that HIV-1 may trigger the release of type I IFN by pDCs, inducing Siglec-1 expression on resident and migrating myeloid DCs in lymphoid tissues, and enhancing viral *trans*-infection to $CD4^+$ T cells (**Figure 50**).

Importantly, our *ex vivo* results in human tonsillar cells have been recently confirmed in murine models, where both HIV-1 and murine leukemia virus (MLV) were used to study retroviral infection [242]. Using different mouse infection models, Sewald et al. visualized how Siglec-1^+ cells disseminate retrovirus in lymphoid tissues of living mice. They demonstrated that HIV-1 and MLV were captured by Siglec-1^+ macrophages from lymph nodes and spleen in a Siglec-1-dependent manner in a humanized BLT mice model. Blockade of Siglec-1 prior to HIV-1 infusion resulted in a lowered viral *trans*-infection to splenocytes [242]. These results demonstrated that retrovirus captured via Siglec-1 promotes efficient retroviral infection *in vivo*. Furthermore, they observed that MLV-containing macrophages could *trans*-infect B-1 cells spreading viral infection within the lymph node [242]. In summary, Siglec-1 expressed on myeloid cells is able to mediate viral *trans*-infection in lymphoid tissues favoring viral dissemination, and could therefore contribute to HIV-1 disease progression.

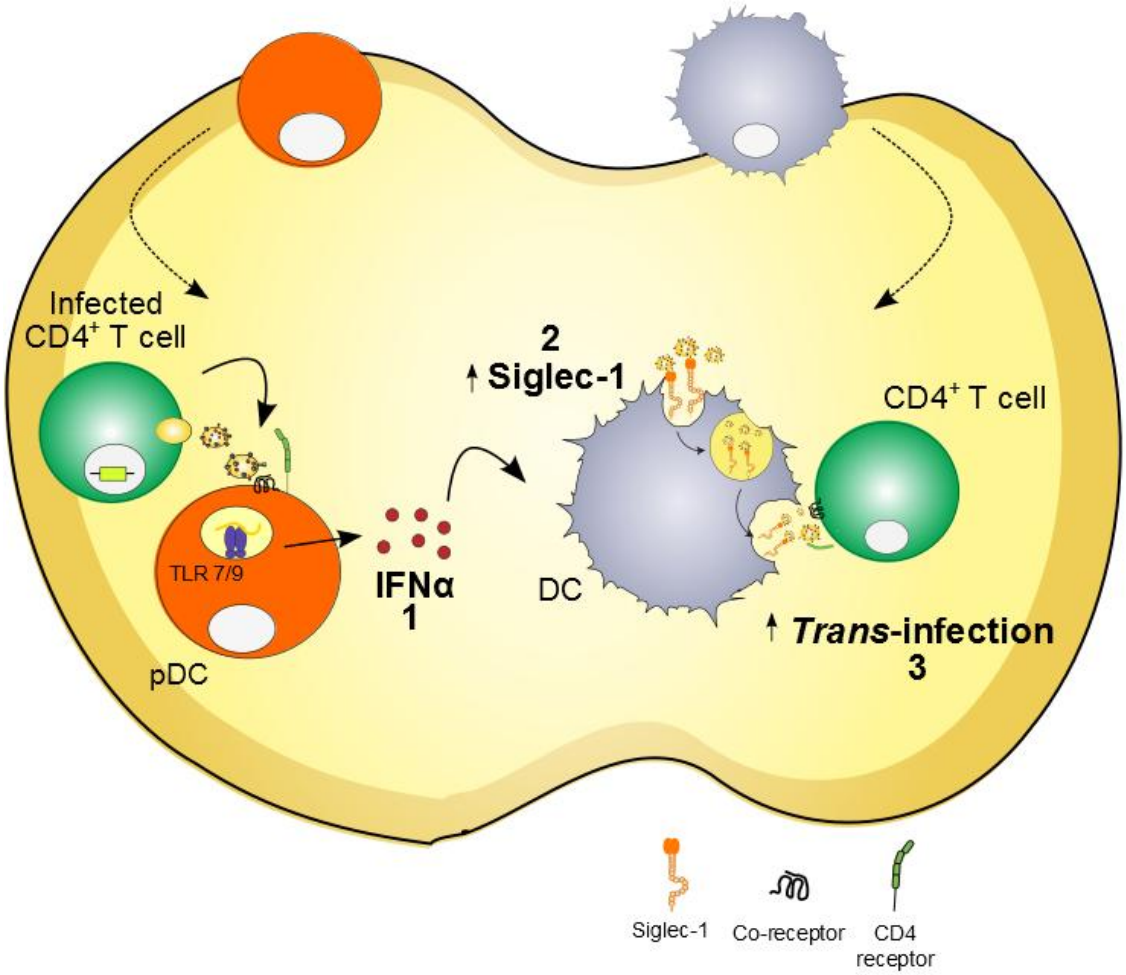


Figure 50. Schematic representation of HIV-1 dissemination in the lymphoid tissue. pDCs and myeloid DCs migrate to lymphoid tissues upon HIV-1 infection. HIV-1-exposed pDCs release IFNα (1), which in turn will induce Siglec-1 on myeloid cells (2), favoring HIV-1 *trans*-infection to bystander $CD4^+$ T cells (3).

5. Siglec-1 blocking compounds as a new strategy to combat HIV-1 cell-to-cell transmission mediated by myeloid cells

Actual antiretroviral treatment is insufficient to cure HIV-1 and the discontinuation of this therapy leads to rebound of detectable viremia [243]. Thus, although HAART blocks viral replication, residual viral replication still persists, probably fueled by cell-to-cell transmission. Finding novel therapeutic approaches aimed at reducing viral spread and limiting the settlement of viral reservoirs should bring us closer to a functional cure of HIV-1. Although others and we have demonstrated that Siglec-1 is an important myeloid cell receptor that augments viral dissemination, no current antiviral therapy blocks this particular pathway. Thus, we believe that designing a novel

generation of therapeutic agents against HIV-1 by targeting Siglec-1 could pose an interesting approach to improve the efficacy of current antiretroviral treatments (**Figure 51**).. Since Siglec-1 expression is augmented on myeloid cells during the acute phase of HIV-1 infection (concomitant to the release of immune activation signals), therapies to block Siglec-1 should be preferentially introduced during this early phase, when myeloid cells are activated and their viral uptake capacity is increased. Furthermore, if Siglec-1 is blocked prior the migration of myeloid cells to lymphoid tissues, it would prevent further viral spread in these sites. Although HIV-1 infection is normally diagnosed during chronic phases, early introduction of HAART has demonstrated superior efficacy compared to delayed initiation of HAART [244, 245], providing the rational for the implementation of early treatment along with prospect Siglec-1 blocking agents.

Potential adverse effects should be taken into account when targeting Siglec-1 or any other human protein. However, Siglec-1 is a cell adhesion receptor whose function might be redundant with that of many other molecules. Furthermore, Siglec-1 knockout mice only exhibit subtle changes in B- and T-cell populations, but are viable, fertile and have no developmental abnormalities [246], indicating that Siglec-1 might be a safe therapeutic target. Moreover, compounds against a human receptor involved in viral membrane ganglioside recognition (derived from the cell host membrane) might have higher genetic barrier to drug resistance, as opposed to other antiretroviral treatments targeting highly mutagenic viral proteins that lead to the appearance of drug-resistant viral scape mutants [247].

In the search for Siglec-1 blocking compounds, monetary costs, efficacy and pharmacokinetic properties are all important variables to take into account. On one hand, high-throughput screening of small-molecules expedites and reduces the cost of the discovery procedure [248]. Because small-molecule compounds are in general orally administered, their plasma concentration vary between patients and interindividual differences in drug responses are observed [200, 249]. On the other hand, mAb development requires relatively complex processes with huge monetary costs compared with small-molecule inhibitors [248]. However, the emergence of humanization techniques of mAb has facilitated this procedure [250, 251]. mAbs are

large proteins that are generally administered intravenously once a week, presenting stable plasma concentrations and long half-lives [200, 249]. Furthermore, mAbs have an efficient biodistribution in different tissues: for instance, clinically approved mAbs targeting B-cells of lymphomas effectively reach lymphoid organs [252, 253]. Despite the advantages that mAbs present, they can trigger immune-effector responses, such as antibody-dependent and complement-dependent cytotoxicity that are not usually triggered by small-molecule inhibitors [254].

Here, we have embraced both small-molecule and mAb discovery strategies in order to maximize the chances of finding a Siglec-1 blocking compound. Specifically, we have followed three different approaches based on the use of small sialyllactose-like agents, multivalent sialyllactose-like compounds, and mAbs against Siglec-1. *In silico* high-throughput screening and docking modeling of Siglec-1 binding to sialyllactose helped us to identify 23 sialyllactose-like compounds with Siglec-1 blocking potential. VLP capture assays demonstrated that three compounds were able to block Siglec-1 viral capture with the same efficacy as sialyllactose. However, high concentrations of these compounds were needed to achieve effective blocking activity (**Figure 38**), rendering these molecules difficult to use in clinical settings. Siglec-1 receptor binds sialylated ligands with low affinity and indeed dissociation constants are in the mM range [138]. In the case of HIV-1, high-affinity of Siglec-1 adhesion is achieved from interactions of thousand of sialyllactose-containing gangliosides in the viral membrane [255, 256] that supports high avidity viral interactions with Siglec-1 receptors clustered on the plasma membrane. These multiple interactions yield stable viral attachment despite the relatively poor affinity of each individual interaction. Thus, strategies to block the high avidity interactions between Siglec-1 and HIV-1 will require the chemical design of compounds with multivalent binding capacity.

Here, we tested two different multivalent structures targeting Siglec-1 receptor: one composed of nine sialic acids and another one displaying three sialyllactose moieties. Neither of these structures were able to block VLP capture (**Figure 39**). The presence of nine sialic acids or three sialyllactose moieties could not surpass a minimum threshold necessary to overcome low-affinity binding interactions. A previous study used similar structures and successfully blocked the C-type lectin receptor DC-SIGN [257]. In this

study, distinct compounds incorporating six to eighteen mannose-like moieties (the natural ligand of DC-SIGN) could inhibit both HIV-1 and Dengue virus infection at low µM concentrations [257]. Differences in blocking susceptibility of multivalent structures between DC-SIGN and Siglec-1 can be attributed to the lower dissociation constant estimated for DC-SIGN (in the nM range) [258], compared with that of Siglec-1 (in the µM to mM range) [138]. Currently we are working to design sialyllactose nanogels, which are scaffolds of polyethyleneglycol that can display a higher number of sialyllactose moieties to increase binding affinity, and consequently, effectively block Siglec-1 mediated viral capture.

Our last strategy to block Siglec-1 receptor relied on the generation of a mAb that could specifically recognize the sialic acid binding site of Siglec-1 within the V-Set domain. Commercial mAbs against Siglec-1 successfully block Siglec-1 receptor *in vitro* [135], however, their Fab regions had no capacity to block VLP capture by Siglec-1 [135]. Thus, Siglec-1 blockage by commercial mAbs most likely occurs due to steric hindrance that prevents viral recognition. Indeed, we tried to produce Siglec-1 blocking mAbs directed only to the specific HIV-1 binding site in the V-Set domain, which we aimed that could be employed for human administration. First, we immunized mice with selected synthetic peptides from the V-Set domain, which were able to develop Abs against their respective linear immunogenic selected peptides (**Figure 41**). However, the Abs produced did not present any Siglec-1 blocking activity (**Figure 42**). These experiments suggest that blocking mAbs require 3D immunogens that favor the recognition of the 3D-structure of the protein to effectively allow Siglec-1 blockade. Thus, by using the whole V-set domain of Siglec-1 as an immunogen, which would maintain the protein structure, we expect to increase the chances of producing Siglec-1 blocking Abs. By creating a DNA construction that contained the peptide leader plus the V-Set domain of Siglec-1, we pursued to produce a recombinant protein of the V-Set domain that could be secreted in the supernatant, facilitating subsequent protein purification [259]. However, the presence of the peptide leader did not result in protein secretion (**Figure 45**). Intuitively, the best peptide leader may arise from the native protein [260]. Nevertheless, some proteins need additional sequences for targeting the plasma membrane [261]. Thereby, Siglec-1 might require the presence of some domains that

were not included in our construction. Protein production and immunization strategies are currently being reformulated to account for these limitations, and we are also exploring the possibility of humanizing commercial mouse mAbs that have proved Siglec-1 blocking capacity in human cells [135].

In conclusion, this thesis supports the importance of Siglec-1 receptor expressed in the myeloid cell lineage and its contribution to HIV-1 pathogenesis. High viremia present during HIV-1 infection triggers the production of different immune activation factors, such as IFNα, which is mainly released by pDCs upon viral exposure. IFNα is an antiviral cytokine that blocks viral replication, but is also one of the main drivers of HIV-1 progression to AIDS. We have observed that IFNα is able to up-regulate Siglec-1 on myeloid cells and contribute to viral spread. Based on our results, this mechanism might be driven by IFNα-activated monocytes and DCs, which exhibited higher Siglec-1 dependent *trans*-infection than macrophages.

This model is consistent with our findings that *in vivo,* Siglec-1 expression correlates with the levels of plasma viral load and diminishes after effective antiretroviral treatment. Furthermore, Siglec-1 is expressed in lymphoid tissues in an inflammation-dependent manner, where it can mediate HIV-1 *trans*-infection. Our results strongly support that Siglec-1 is an important molecule that could accelerate HIV-1 transmission in the crowded cellular environment of lymphoid tissues, where many T cells can contact myeloid cells. The discovery of the role of Siglec-1 in capturing HIV-1 expands our understanding of HIV-1 transmission mechanisms and highlights the contribution of myeloid cells to HIV-1 pathogenesis. The different strategies pursued in this thesis to generate Siglec-1 blocking compounds have allowed us to optimize cellular and tissular culture models in which to perform future screenings of prospect Siglec-1 blocking candidates. Gathered knowledge will help us to design prospect therapeutic approaches aimed to prevent viral dissemination and implement current antiretroviral treatments.

Chapter 8

CONCLUSIONS

Objective 1: To determine the expression of Siglec-1 on IFNα activated myeloid cells such as monocytes and macrophages and compare it to that of DCs in order to assess if immune activation stimuli present during HIV-1 infection can trigger Siglec-1 induction and mediate viral capture and *trans*-infection.

1. 1. Siglec-1 is up-regulated on all myeloid cells upon IFNα treatment and mediates HIV-1 capture.

1. 2. Siglec-1 mediates viral uptake into a storage compartment and enhances HIV-1 *trans*-infection, especially in IFNα-treated monocytes and DCs.

1. 3. IFNα antiviral activity does not inhibit Siglec-1 mediated HIV-1 transmission to $CD4^+$ T cells.

2. Objective: To elucidate if immune activating signals present in plasma from untreated HIV-1-infected patients could induce Siglec-1 expression and contribute to HIV-1 pathogenesis by modulating trans-infection.

2. 1. Siglec-1 is up-regulated on monocytes from HIV-1-infected individuals, and its expression is reduced upon successful antiretroviral treatment.

2. 2. The plasma of untreated HIV-1-infected individuals stimulates Siglec-1 expression and signals via the type I IFN receptor.

2. 3. Expression of Siglec-1 on monocytes positively correlates with viral load and negatively correlates with CD4+ T cell counts.

3. Objective: To assess whether IFNα stimulation could induce Siglec-1 on myeloid cells isolated from lymphoid tissues, and consequently, contribute to viral spread throughout the course of HIV-1 infection.

3. 1. Immunohistochemical stains show that Siglec-1 positive cells accumulate in inflamed lymphoid tissues within areas enriched in $CD4^+$ T cells.

3. 2. Cells isolated from lymphoid tissues exposed to IFNα express Siglec-1

3. 3. Transcriptomic analyses indicate that Siglec-1 expressing cells isolated from lymphoid tissues present a unique myeloid antigen presenting cell profile.

3. 4. Siglec-1 expressed on myeloid cells isolated from lymphoid tissue mediates HIV-1 capture and storage in a sac-like compartment.

3. 5. Siglec-1 enhances HIV-1 *trans*-infection in myeloid cells isolated from lymphoid tissue.

4. Objective: To develop sialyllactose-like small compounds (in monovalent and multivalent formulations) and specific mAbs against the V-Set domain that could effectively block viral capture and cell-to-cell transmission mediated by Siglec-1.

4.1. Small sialyllactose-like molecules and sialyllactose-containing glycodendrons do not block Siglec-1-mediated viral capture.

4.2. Recognition of small immunogenic Siglec-1 peptides by antibodies from immunized mice is not sufficient to block Siglec-1 recognition of HIV-1.

Chapter 9

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Chapter 11

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