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# Human Immunodeficiency Virus Type I subverts T cell extracellular matrix to shelter cell-associated infectivity in a viral biofilm

Catherine Inizan

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**Thèse de Doctorat de l'Université Pierre et Marie Curie – Paris VI**  
École Doctorale ED394 – Physiologie, Physiopathologie

Présentée par  
**Catherine Inizan**

Pour obtenir le titre de Docteur de l'Université Pierre et Marie Curie – Paris VI

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Soutenue publiquement le 7 Juillet 2015, devant le jury composé de :

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**Dr Jean-Christophe PAILLART**  
**Prof Uriel HAZAN**  
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**Dr Arnaud MORIS**  
**Dr Maria-Isabel THOULOZE**

**Président**  
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## List of abbreviations

3TC	: 2',3'-dideoxy-3'-thiacytidine Lamivudine (commercial name Epivir®)
AAV	: <u>A</u> deno- <u>A</u> ssociated <u>V</u> irus
ABC	: Abacavir
ADCC	: <u>A</u> ntibody- <u>D</u> ependent <u>C</u> ell-mediated <u>C</u> ytotoxicity
ADCVI	: <u>A</u> ntibody- <u>D</u> ependent <u>C</u> ellular <u>V</u> irus <u>I</u> nhibition
AID	: <u>A</u> ctivation-induced <u>C</u> ytidine <u>D</u> eaminase
AIDS	: <u>A</u> cquired <u>I</u> mmunodeficiency <u>S</u> ndrome
APC	: <u>A</u> ntigen <u>P</u> resenting <u>C</u> ell
AP 1	: <u>A</u> ctivator <u>P</u> rotein 1
ArT	: <u>A</u> ntiretroviral <u>T</u> reatment
ASC	: <u>A</u> ntibody- <u>S</u> ecreting <u>C</u> ells
AZT	: <u>A</u> zidothymidine (Zidovudine ZDV, commercial name Retrovir®)
bNAbs	: <u>b</u> roadly <u>N</u> eutralizing <u>A</u> ntibodies
CA	: viral <u>C</u> apsid protein
CAp24	: viral <u>C</u> apsid protein 24 kDa
CCR5	: <u>C</u> - <u>C</u> chemokine <u>R</u> eceptor type 5
CDn	: <u>C</u> luster of <u>D</u> ifferentiation n ( $n \in \mathbb{N}$ )
CD45RO	: <u>C</u> luster of <u>D</u> ifferentiation 45 Isoform RO
CDC	: <u>C</u> enter of <u>D</u> isease <u>C</u> ontrol
C/EBP $\beta$	: <u>C</u> CAAT/ <u>E</u> nhancer- <u>B</u> inding <u>P</u> rotein <u>b</u> eta
CFP	: <u>C</u> yan <u>F</u> luorescent <u>P</u> rotein
CMV	: <u>C</u> ytomegalovirus
CTD	: <u>C</u> - <u>T</u> erminal <u>D</u> omain
CTL	: <u>C</u> ytotoxic <u>T</u> <u>L</u> ymphocyte
CXCR4	: <u>C</u> - <u>X</u> - <u>C</u> chemokine <u>R</u> eceptor type 4
DC	: <u>D</u> endritic <u>C</u> ell
Dlg1	: <u>D</u> isks <u>l</u> arge homolog 1
DNA	: <u>D</u> eoxyribonucleic <u>A</u> cid
dsDNA	: <u>d</u> ouble- <u>s</u> tranded <u>D</u> eoxyribonucleic <u>A</u> cid
ECM	: <u>E</u> xtracellular <u>M</u> atrix
EFV	: <u>E</u> favirenz (commercial name Sustiva®)

eIF2	: eukaryotic <u>I</u> nitiation <u>F</u> actor 2
ELISA	: <u>E</u> nzyme- <u>L</u> inked <u>I</u> mmunosorbent <u>A</u> ssay
Env	: viral <u>E</u> nvelope protein
Erk1/2	: <u>E</u> xtracellular signal- <u>r</u> egulated <u>k</u> inase 1/2
ESCRT	: <u>E</u> ndosomal <u>S</u> orting <u>C</u> omplexes <u>R</u> equired for <u>T</u> ransport
FGF	: <u>F</u> ibroblast <u>G</u> rowth <u>F</u> actor
FDA	: <u>F</u> ood and <u>D</u> rug <u>A</u> dmistration
FIB/SEM	: <u>F</u> ocused <u>I</u> on <u>B</u> eam/ <u>S</u> canning <u>E</u> lectron <u>M</u> icroscopy
FITC	: <u>F</u> luorescein <u>I</u> sothiocyanate
FTC	: Emtricitabine (commercial name Emtriva®)
Gag	: <u>G</u> roup-specific <u>A</u> ntigen, viral capsid protein
GAG	: <u>G</u> lycosaminoglycan
GALT	: <u>G</u> ut- <u>A</u> ssociated <u>L</u> ymphoid <u>T</u> issue
GlcA	: glucuronic acid
GlcNAc	: N-acetyl-glucosamine
Gly	: glycine
GM1	: monosialotetrahexosylganglioside
gp41 <sup>TM</sup>	: glycoprotein 41 <u>T</u> rans <u>M</u> embrane
gp120 <sup>SU</sup>	: glycoprotein 120 <u>S</u> urface
HAART	: <u>H</u> ighly <u>A</u> ctive <u>A</u> ntiretroviral <u>T</u> herapy
HDAC	: <u>H</u> istone <u>D</u> eacetylase
HIV-1, 2	: <u>H</u> uman <u>I</u> mmunodeficiency <u>V</u> irus Type 1, 2
HLA	: <u>H</u> uman <u>L</u> eucocyte <u>A</u> ntigen
hnRNP-K	: heterogeneous nuclear <u>R</u> ibonucleoprotein <u>K</u>
HS	: <u>H</u> eparan <u>S</u> ulfate
HSG	: <u>H</u> uman <u>S</u> alivary <u>G</u> land
HSPG	: <u>H</u> eparan- <u>S</u> ulfate <u>P</u> roteoglycan
HTLV-I	: <u>H</u> uman <u>T</u> Cell <u>L</u> eukemia <u>V</u> irus Type I
HTLV-III	: <u>H</u> uman <u>T</u> Cell <u>L</u> eukemia <u>V</u> irus Type III
HTS	: <u>H</u> igh <u>T</u> hroughput <u>S</u> creening
hu-PBL-SCID	: human <u>P</u> eripheral <u>B</u> lood <u>L</u> ymphocytes - <u>S</u> evere <u>C</u> ombined <u>I</u> mmunodeficiency
IA-SEM	: <u>I</u> on <u>A</u> brasion – <u>S</u> canning <u>E</u> lectron <u>M</u> icroscopy
IC	: <u>I</u> nfected <u>C</u> ell

ICAM-1, 3	: <u>I</u> nter <u>c</u> ellular <u>A</u> dhesion <u>M</u> olecule-1, 3
iDC	: <u>i</u> mmature <u>D</u> endritic <u>C</u> ell
IFN	: <u>I</u> nter <u>f</u> er <u>o</u> n
IgA	: <u>I</u> mmunoglobulin isotype <u>A</u>
iGFP	: <u>i</u> nternal <u>G</u> reen <u>F</u> luorescent <u>P</u> rotein
IgG	: <u>I</u> mmunoglobulin isotype <u>G</u>
IL	: <u>I</u> nter <u>l</u> eukine
IN	: viral <u>I</u> ntegrase
INVS	: <u>I</u> nstitut <u>N</u> ational de <u>V</u> eille <u>S</u> anitaire (National Institut for Health Surveillance)
Lamp-2	: <u>L</u> ysosome- <u>a</u> ssociated <u>m</u> embrane protein 2
LAV	: <u>L</u> ymphadenopathy- <u>A</u> ssociated <u>V</u> irus
LC	: <u>L</u> angehans <u>C</u> ell OR <u>L</u> iquid <u>C</u> hromatography
Lck	: <u>l</u> ymphocyte-specific protein tyrosine <u>k</u> inase
LFA-1	: <u>L</u> ymphocyte <u>F</u> unction-associated <u>A</u> ntigen 1
LTR	: <u>L</u> ong <u>T</u> erminal <u>R</u> epat
MAp17	: <u>M</u> atrix protein 17 kDa
MAPK	: <u>M</u> itogen- <u>A</u> ctivated <u>P</u> rotein <u>K</u> inase
mAb	: <u>m</u> onoclonal <u>A</u> ntibody
mDC	: <u>m</u> ature <u>D</u> endritic <u>C</u> ell
MDM	: <u>M</u> onocyte <u>D</u> erived <u>M</u> acrophage
MFG-E8	: <u>M</u> ilk <u>f</u> at globule <u>E</u> GF factor 8
MHCI or II	: <u>M</u> ajor <u>H</u> istocompatibility <u>C</u> omplex Class I or II
MIP-1 $\beta$	: <u>M</u> acrophage <u>I</u> nflammatory <u>P</u> rotein-1 $\beta$
MMLV	: <u>M</u> oloney <u>M</u> urine <u>L</u> eukemia <u>V</u> irus
MMP	: <u>M</u> atrix <u>M</u> etalloprotease
MOI	: <u>M</u> ultiplicity <u>o</u> f <u>I</u> nfection
MR	: <u>M</u> annose <u>R</u> eceptor
mRNA	: <u>m</u> essenger <u>R</u> ibonucleic <u>A</u> cid
MSM	: <u>M</u> en who have sex with <u>m</u> en
MS/MS	: tandem <u>M</u> ass <u>S</u> pectrometry
MT-MMP	: <u>M</u> embrane- <u>T</u> ype <u>M</u> atrix <u>M</u> etalloprotease
mTOR	: <u>m</u> ammalian <u>T</u> arget <u>o</u> f <u>R</u> apamycin
MVB	: <u>M</u> ultivesicular <u>B</u> ody

MW	: <u>M</u> echanically <u>W</u> ashed
myDC	: <u>m</u> yeloid <u>D</u> endritic <u>C</u> ell
NA	: <u>N</u> - <u>a</u> cetylated
NAbs	: <u>N</u> eutralizing <u>A</u> ntibodies
NASBA	: <u>N</u> ucleic Acid Sequence Based Amplification
NF-κB	: <u>N</u> uclear <u>F</u> actor- <u>κ</u> <u>B</u>
NHP	: <u>N</u> on- <u>H</u> uman <u>P</u> rimate
NI	: <u>N</u> on <u>I</u> nfected
NK	: <u>N</u> atural <u>K</u> iller
NNRTI	: <u>N</u> on- <u>N</u> ucleoside <u>R</u> everse <u>T</u> ranscriptase <u>I</u> nhibitor
NRTI	: <u>N</u> ucleoside <u>R</u> everse <u>T</u> ranscriptase <u>I</u> nhibitor
NS	: <u>N</u> - <u>s</u> ulfated
NTD	: <u>N</u> - <u>T</u> erminal <u>D</u> omain
NW	: <u>N</u> on <u>W</u> ashed
ORF	: <u>O</u> pen <u>R</u> eading <u>F</u> rame
PBMCs	: <u>P</u> eripheral <u>B</u> lood <u>M</u> ononuclear <u>C</u> ells
PCP	: pneumocystis <u>c</u> arini pneumonia
PCR	: <u>P</u> olymerase <u>C</u> hain <u>R</u> eaction ( <u>R</u> éaction de <u>P</u> olymérisation en <u>c</u> haîne)
PDI	: <u>P</u> rotein <u>D</u> isulfide <u>I</u> somerase
pH	: potentiel <u>H</u> ydrogène
PHA	: <u>p</u> hyto- <u>h</u> emagglutinin
PI	: <u>P</u> rotease <u>I</u> nhibitor or <u>P</u> ropidium <u>I</u> odid
PI3-kinase	: <u>p</u> hospho <u>i</u> nositide 3-kinase
PKCdelta	: <u>P</u> rotein <u>K</u> inase <u>C</u> <u>d</u> elta
PKR	: <u>P</u> rotein <u>K</u> inase <u>R</u> NA-activated
PM	: <u>P</u> lasma <u>M</u> embrane
PMA	: <u>P</u> horbol <u>M</u> yristate <u>A</u> cetate
PND	: <u>P</u> rincipal <u>N</u> eutralization <u>D</u> eterminant
PPE	: <u>P</u> ost- <u>E</u> xposure <u>P</u> rophylaxis
PR	: viral <u>P</u> rotease
Pro	: <u>P</u> roline
Pr55 <sup>gag</sup>	: <u>P</u> recursor of 55 kDa for Gag
PVVIH	: <u>P</u> ersonne <u>V</u> ivant avec le <u>V</u> IH (Person living with HIV)
RANTES	: <u>r</u> egulated on <u>a</u> ctivation, <u>n</u> ormal <u>T</u> cell <u>e</u> xpressed and <u>s</u> ecreted (CCL5)

RGD	: arginine glycine aspartate
RM	: <u>R</u> hesus <u>M</u> acaque
RNA	: <u>R</u> ibonucleic <u>A</u> cid
RRE	: <u>R</u> ev <u>R</u> esponsive <u>E</u> lement
RT	: viral <u>R</u> everse <u>T</u> ranscriptase
RT-PCR	: <u>R</u> everse <u>T</u> ranscription – <u>P</u> olymerase <u>C</u> hain <u>R</u> eaction
SAHA	: <u>S</u> uberoylanilide <u>H</u> ydroxamic <u>A</u> cid
SD	: <u>S</u> tandard <u>D</u> eviation
SDF-1	: <u>S</u> tromal cell- <u>D</u> erived <u>F</u> actor 1
SEB	: <u>S</u> taphylococcal <u>E</u> nterotoxin <u>B</u>
SEM	: <u>S</u> tandard <u>E</u> rrors of the <u>M</u> ean or Scanning <u>E</u> lectron <u>M</u> icroscopy
SILAC	: <u>S</u> table <u>I</u> sotope <u>L</u> abeling by <u>A</u> mino acids in <u>C</u> ell culture
siRNAs	: short interfering <u>R</u> NAs
SIV	: <u>S</u> imian <u>I</u> mmunodeficiency <u>V</u> irus
SM	: <u>S</u> ootey <u>M</u> angabey
SN	: <u>S</u> upernatant
SP-1	: <u>S</u> pecificity <u>P</u> rotein 1
Subt	: <u>S</u> ubtilisin
SW	: <u>S</u> ingle <u>W</u> ash
T20	: Enfuvirtide (molecule Fuzeon)
TAR	: <u>T</u> rans-activation <u>R</u> esponse element
TEM	: <u>T</u> ransmission <u>E</u> lectron <u>M</u> icroscopy
TC	: <u>T</u> arget <u>C</u> ell
TCGF	: <u>T</u> -cell growth factor
TDF	: <u>T</u> enofovir <u>D</u> isoproxil <u>F</u> umarate (commercial name Viread®)
TGFβ	: <u>T</u> ransforming <u>G</u> rowth <u>F</u> actor <u>β</u>
TLRs	: <u>T</u> oll <u>L</u> ike <u>R</u> eceptors
TNF	: <u>T</u> umor <u>N</u> ecrosis <u>F</u> actor
TSG101	: <u>T</u> umor <u>s</u> usceptibility gene 101
TSLP	: <u>T</u> hymic <u>S</u> tromal <u>L</u> yphopoietin
USA	: <u>U</u> nited <u>S</u> tates of <u>A</u> merica
VCAM	: <u>V</u> ascular <u>C</u> ell <u>A</u> dhesion <u>M</u> olecule 1
VS	: <u>V</u> irological <u>S</u> ynapse
WHO	: <u>W</u> orld <u>H</u> ealth <u>O</u> rganization

WT : Wild Type  
YFP : Yellow Fluorescent Protein  
ZDV : Zidovudine









## Preamble

With 35 million reported cases worldwide, HIV/AIDS represents the pandemic of the century. Within its host, HIV-1 mostly replicates in T cells and HIV-1 transmission by cell contacts between T cells represents an efficient mechanism of viral dissemination. In this context, understanding the nature of the infectious material transferred at intercellular junctions is a central and unexplored subject of fundamental research.

The work presented in this manuscript enabled the characterization of HIV-1 biofilm as the main source for T-cell associated infectivity. More precisely, this study initiates the description of the morphology of HIV-1 biofilm and provides some insights into its functional features potentially contributing to the pathophysiology of AIDS.



# **INTRODUCTION**



# I. AIDS worldwide today

## 1. Natural history of a pandemic

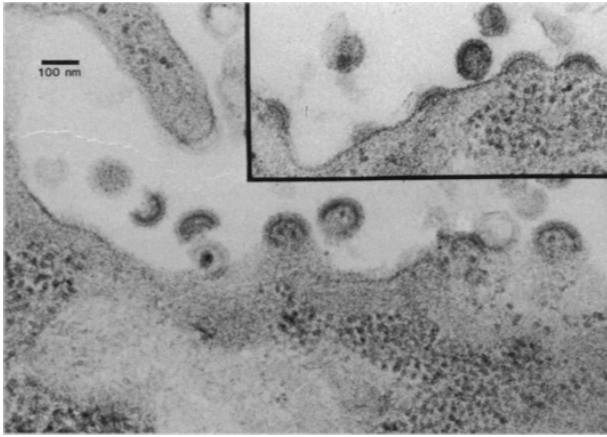
The history of Acquired Immunodeficiency Syndrome (AIDS) dates to June 1981 when the Center for Disease Control (CDC) reported the unprecedented occurrence of pneumocystis carini pneumonia (PCP) in five previously healthy homosexual men, all five of whom had documented cytomegalovirus (CMV) infections and reported use of inhalant drugs (Center for Disease Control, 1981). Soon thereafter, numerous reports began to appear of the alarming occurrence of PCP as well as other opportunistic infections and Kaposi's sarcoma in previously healthy homosexual males. Over the ensuing year, increasing numbers of recognized cases have been reported to CDC, the population at risk expanding to include heterosexual men and women.

In November 1982, 691 cases were reported, yielding a devastating mortality rate of 40%. A disordered immunoregulation, called Acquired Immunodeficiency Syndrome (AIDS), was consistently found in all patients described, involving T-lymphocyte hyporesponsiveness and imbalance in T-helper and suppressor cells. At the time, the underlying factor responsible for this immunoregulatory defect was unknown but possible etiologies included chronic antigen stimulation, a transmissible infectious agent, drug use, and spermatozoa exposure (Quagliarello, 1982).

In May 1983, while 3,064 cases of AIDS are reported to CDC (Center for Disease Control, 1983), a research team from the Institut Pasteur in Paris identifies from a lymph node of a homosexual patient with multiple lymphadenopathies a «retrovirus belonging to the family of recently discovered human T-cell leukemia viruses (HTLV), but clearly distinct from each previous isolate » as the possible etiological agent for AIDS (Barre-Sinoussi et al., 1983). Acknowledged by a Nobel prize in Medicine in 2008 for their discovery, Françoise Barré-Sinoussi, Luc Montagnier and collaborators described the horizontal transfer of the virus, its ability to bud from the plasma membrane of infected T lymphocytes as shown in Figure 1, its preference for magnesium for its reverse transcriptase activity as well as the presence of an internal antigen p24/p25 (Barre-Sinoussi et al., 1983). This virus was first called LAV for Lymphadenopathy-Associated Virus. In 1984, the group of Robert Gallo described a correlation between exposure to Human T Cell Leukemia Virus Type III (HTLV-III) and AIDS (Markham et al., 1984). A few months later, HTLV-III and LAV proved to be



the same virus and in 1986, a subcommittee of the International Committee on Taxonomy of Viruses recommended that the retrovirus isolate identified as the causative agent for AIDS be renamed human immunodeficiency virus, to be abbreviated HIV, a taxonomy soon adopted by the world scientific community (Coffin et al., 1986).



**Figure 1. Transmission Electron Microscopy of LAV budding from the plasma membrane of T lymphocytes.**

*Virus was amplified from the biopsy of a cervical lymph node from a 33-year-old homosexual patient presenting lymphadenopathy and asthenia. Cells from the biopsied lymph node were first cultured with phyto-hemagglutinin (PHA), T-cell growth factor (TCGF) and antiserum to human interferon (IFN). Coculture with lymphocytes of*

*the biopsy enabled the transmission of the virus to T lymphocytes isolated from an adult healthy donor. Cell-free supernatant of this infected coculture was used to further infect T lymphocytes from umbilical cords in the presence of Polybrene (2 ug/ml). Electron microscopy of the infected umbilical cord lymphocytes showed characteristic immature particles with dense (C-type) crescent budding at the plasma membrane (Barre-Sinoussi et al., 1983).*

Soon after the identification of AIDS in humans, outbreaks of simian AIDS were identified in captive colonies of Asian rhesus macaques, leading to the discovery of a related retrovirus termed Simian Immunodeficiency Virus (SIV) (Daniel et al., 1985). This finding opened new avenues for fundamental research and vaccine development in Non-Human Primate (NHP) models. A few years later, a virus more closely related to SIV than HIV-1 was isolated from West African AIDS patients (Clavel et al., 1986). This related retrovirus, now known as HIV-2, causes asymptomatic infection in most patients in link with an enhanced immune response and a lower viral replication (for review see (de Silva et al., 2008)).

This work exclusively focuses on HIV-1, although extending our study to a related virus with a different physiopathology such as HIV-2 would be of high interest.

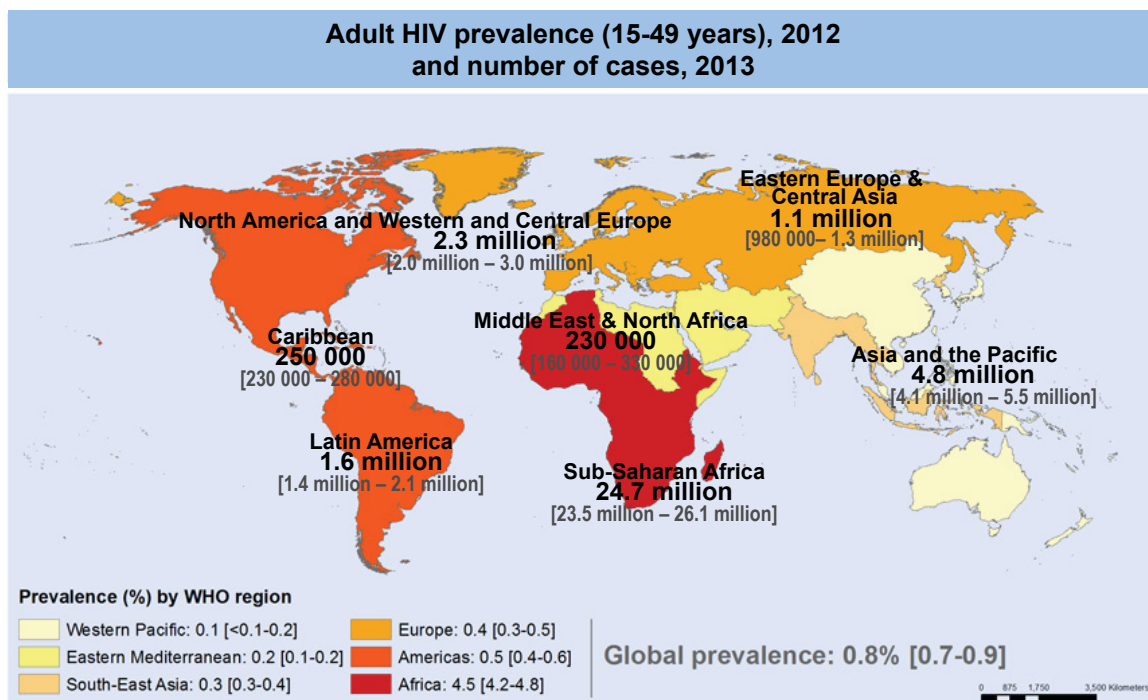
Nowadays, over 30 years after the identification of the first cases, HIV-1 continues to be a major global public health issue, having infected 75 million people and claimed more than 39 million lives so far. In 2013, the World Health Organization (WHO) reported 35 million people living with HIV, representing an estimated 0.8 % of adults aged 15–49 years, 2.1 million people newly infected and 1.5 million deaths caused by AIDS-related illness (World Health Organization, 2013), as shown in Table 1.

**Table 1. Recent figures of HIV pandemy.**

<b>Number of people living with HIV in 2013</b>	<b>Total</b>	<b>35.0 million</b>	[33.1 – 37.2 million]
	<b>Adults</b>	31.8 million	[30.1 – 33.7 million]
	<b>Women</b>	16.0 million	[15.2 – 16.9 million]
	<b>Children (&lt;15 years)</b>	3.2 million	[2.9 – 3.5 million]
<b>People newly infected with HIV in 2013</b>	<b>Total</b>	<b>2.1 million</b>	[1.9 – 2.4 million]
	<b>Adults</b>	1.9 million	[1.7 – 2.1 million]
	<b>Children (&lt;15 years)</b>	240,000	[210,000 – 280,000]
<b>AIDS deaths in 2013</b>	<b>Total</b>	<b>1.5 million</b>	[1.4– 1.7 million]
	<b>Adults</b>	1.3 million	[1.2 – 1.5 million]
	<b>Children (&lt;15 years)</b>	190,000	[170,000 – 220,000]

*From (World Health Organization, 2013)*

Although globally affecting the entire planet, the overall repartition of HIV infections is rather unequal. While Eastern Europe, Central Asia and Pacific seem rather spared by the epidemic, with a prevalence below 0.1 % in Western Pacific, Sub-Saharan Africa is the most affected region, with 24.7 [23.5–26.1] million people living with HIV in 2013, accounting for almost 70 % of global HIV infections, as shown in Figure 2. Developed countries such as North America and Western and Central Europe also carry the burden of HIV infections, with 2.3 [2.0–3.0] million people infected (World Health Organization, 2013). In 2012 in France, 150,000 persons live with HIV (Personnes Vivants avec le VIH, PVVIH) and 6,372 persons have discovered their serological status (INVS, 2013).



**Figure 2. World map of HIV prevalence and number of cases.**

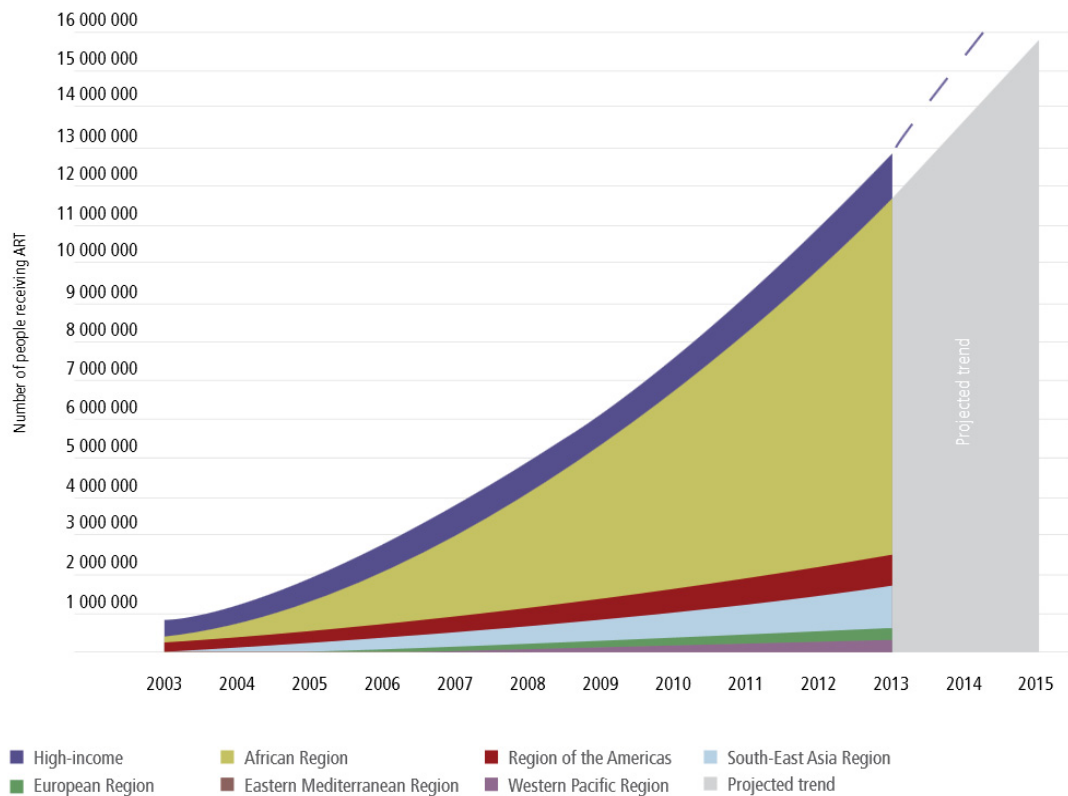
*World map of HIV prevalence by WHO regions among adults between 15 and 49 years old, showing a prevalence in percent ranging from 0.1 % in Western Pacific to 4.5 % in Africa, yielding a global world prevalence of 0.8 %. For further clarity, HIV number of cases per sub-region has been added, displaying 1.1 million cases in Eastern Europe and Central Asia, while Sub-Saharan Africa claims 24.7 million cases, accounting for almost 70 % of HIV world cases. Developed countries are also affected by HIV pandemy, with 2.3 million cases and a prevalence ranging from 0.3 to 0.6 % in North America and Western and Central Europe (from (World Health Organization, 2013)).*

While 28 million people worldwide are eligible for treatment according to WHO recommendations 2013, only 13 million people have access to antiretroviral treatment (ArT) (World Health Organization, 2013), as shown in Figure 3, and very few countries have an ArT coverage above 80 % (World Health Organization, 2013). Yet, as a person with suppressed HIV viremia is very unlikely to transmit the virus (UNAIDS, 2014), prevention benefits of ArT are apparent at the population level. For instance, in KwaZulu-Natal, South Africa, or in the Canadian province of British Columbia, every 1 % increase in coverage yields a 1.1-1.2 % reduction in HIV incidence (UNAIDS, 2014). Rapid expansion of ArT to all people living with HIV is therefore projected to avert 3.3 million new HIV infections in South Africa through 2050 (UNAIDS, 2014).

In addition, studies demonstrate that since the launch of WHO 2013 guidelines, people receiving early treatment (CD4 count 250-500) have higher median CD4 counts during two years of follow-up, are 27 % less likely to experience a primary clinical event, 36 % less likely to experience an AIDS-defining clinical event and 51 % less likely to be diagnosed with tuberculosis. Whereas someone who acquired HIV in the pre-treatment era could expect to live only 12.5 years, a young person in industrialized countries who becomes infected today can expect to live a near normal lifespan (or an additional five decades) with the use of lifelong, uninterrupted HIV treatment.

Increasing ArT coverage is thus beneficial both at the population and at the individual level. UNAIDS has therefore planned an ambitious target for 2020:

- 90 % of all people living with HIV will know their HIV status
- 90 % of all people with diagnosed HIV will receive sustained ArT
- 90 % of all people receiving ArT will have viral suppression.



**Figure 3. Actual and projected numbers of people receiving ArT, 2003-2015.**

*The number of people receiving ArT has continuously increased over the last 12 years, reaching 13 million in 2013. However, in many countries worldwide, ArT coverage is still below 80 %. From (World Health Organization, 2013).*

## 2. Physiopathology of AIDS

### a. Modes of contamination

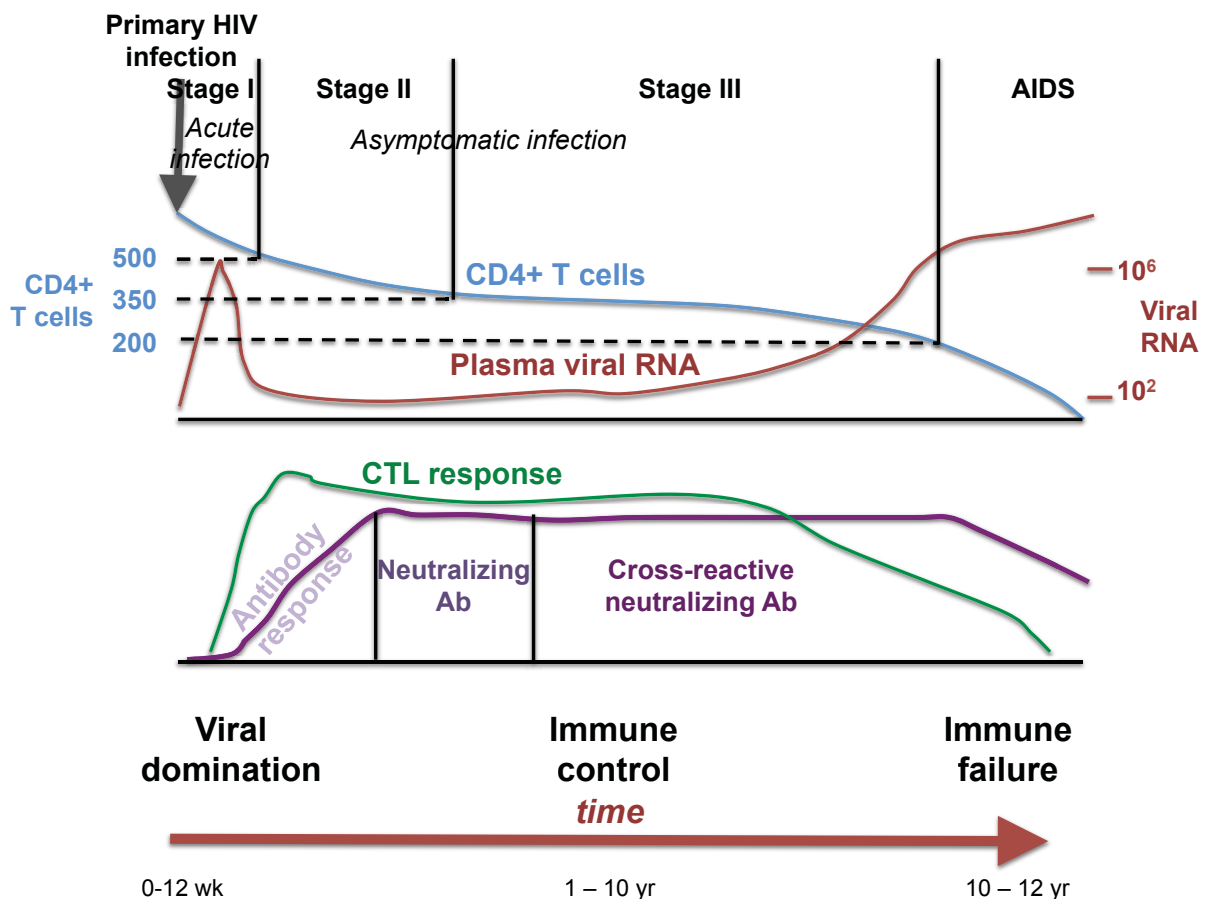
Although relatively inefficient at transmission with an estimated one contamination per 200 to 2,000 contacts (Powers et al., 2008), HIV-1 can be transmitted from one individual to another upon exchange of body fluids containing a sufficient amount of virus (Quinn et al., 2000). HIV-1 can indeed be detected as cell-free viral particles or carried by infected cells in a variety of body fluids such as blood (Daar et al., 1991; Re et al., 1994), breast milk (Homsy et al., 1988; Van de Perre et al., 1992), vaginal secretions (Wofsy et al., 1986) and semen (Bagasra et al., 1994). In the semen, spermatozoa themselves are able to carry HIV-1 virions at their surface without getting infected (Dussaix et al., 1993), either through interaction with HIV-1 coreceptor CCR5 they express (Muciaccia et al., 2005) or through interaction with heparan sulfates (Ceballos et al., 2009).

Horizontal transmissions through blood (drug addiction, accidents, blood transfusions) or through sexual contact are thus the common modes of contamination. Vertical transmission is also possible from mother to child during pregnancy, at childbirth or during breast feeding (World Health Organization, 2013).

Therefore, populations considered to be at risk for HIV contamination are drug users (28 % of HIV-1 prevalence among drug users in Asia), men who have sex with men (MSM) (over 1 % of HIV-1 prevalence among MSM worldwide) and sex workers (who are 13.5 times more likely to be living with HIV-1 than other women) (World Health Organization, 2013). Aware of the struggles of those key populations, WHO intends to halve the transmission of HIV among people who inject drugs and to reduce sexual transmission of HIV by 50 % by 2015.

### b. Symptoms and stages of the disease

According to CD4 counts, evolution of the viral load, symptoms and set up of an immune response, HIV infection can be staged using 4 categories, from Stage I to Stage IV, as exposed in Figure 4.



**Figure 4. Staging and evolution of virological and immune parameters over the course of HIV infection.**

Following **Primary HIV-1 infection**, **stage I** establishes. CD4 count is **above 500/ $\mu$ L** and plasmatic viremia may exceed 10,000 copies per mL (Daar et al., 1991). This early period of infection represents the time of greatest infectivity and may involve lymph node enlargement and mononucleosis-like symptoms (weight loss, fever, diarrhea, cough). Initial non-neutralizing anti-gp41 and anti-gp120 antibody response appears  $\sim$ 1 week post-infection (Mouquet, 2014).

**Stage II** begins when CD4 counts drop **below 500/ $\mu$ L**. Viral replication is still active but plasmatic viremia declines about 100 fold (Daar et al., 1991). Patients display mild symptom (mucocutaneous manifestations, upper respiratory tract infections). Autologous neutralizing antibodies appear several months post-infection. Crossreactive neutralizing antibodies (neutralizing heterologous viruses) generally develop 2 to 4 years after infection.

**Stage III** initiates when CD4 counts drop **below 350/ $\mu$ L**. Viral load increases and patients experience clinical immunodeficiency associated to advanced symptoms and opportunistic infections (HCV: 4 to 5 million coinfections (Sherman et al., 2014), tuberculosis: 13 % of tuberculosis patients are infected with HIV-1 (World Health Organization, 2013)).

**Stage IV or AIDS** develops 2 to 15 years after infection, when CD4 count drops **below 200/ $\mu$ L**. AIDS is associated to severe opportunistic infections and comorbidities (toxoplasmosis, candidiasis, meningitis and cancers) (from (Mosier, 2000)).

Overall, an inverse correlation between CD4 counts and viral load on the one hand (Venet et al., 1991) and between CD4 counts and fraction of infected CD4 T cells on the other hand (Bruisten et al., 1993) is observed throughout the course of the disease. If one needs to

optimize the probabilities of observing a fair amount of infected CD4 T cells in blood samples from patients, a compromise has to be found between high viral load and high enough CD4 counts.

### c. Anti-HIV-1 immune response

The host's immune response kicks in as soon as Stage II, inducing a decline in the level of virus in the blood. This immune response involves both innate and adaptive arms, leading to cellular and humoral responses.

Innate sensing pathways can potentially detect every early step of HIV-1 retroviral life cycle. HIV-1 entry can hence be detected by Toll-Like receptors (TLRs). For instance, HIV-1 gp120 activates TLR9 (Martinelli et al., 2007) while viral nucleic acids from degraded or defective incoming particles are sensed by TLR7 (Kane et al., 2011) and 8 (Gringhuis et al., 2010). In addition, incoming capsid lattice is detected by cytoplasmic TRIM5 (Pertel et al., 2011). Later in HIV-1 life cycle, reverse-transcribed HIV DNA triggers recognition by cyclic AMP-GMP synthase (Gao et al., 2013). Lastly, proviral integration into chromosomal DNA may trigger the DNA damage response pathway mediated by DNA-PK (Cooper et al., 2013). Innate sensing pathways ultimately lead to cell death by pyroptosis (Doitsh et al., 2014) or to IFN secretion. Type I IFN secretion by DCs, and especially pDCs, in turn activates NK cells that rapidly expand and promote early viral control following acute infection (Alter et al., 2007).

Adaptive cellular and humoral responses are initiated by DCs and macrophages that phagocytose, process and present viral determinants, thus promoting the activation of anti-HIV-1 T-Helper CD4<sup>+</sup> T cells. Adaptive cellular response involves cytotoxic CD8<sup>+</sup> T lymphocytes (CTLs) that target epitopes exposed at the surface of HIV-1 infected T cells. CTLs secrete pro-inflammatory cytokines such as IL-2 and IFN- $\gamma$  and kill HIV-1 infected cells through targeted release of granzyme B and perforin. As CTL appearance correlates with viral decline, and higher CTL numbers correlate with lower viremia, CTLs appear essential for the decline in viral load observed at Stages II and III (Mosier, 2000).

Humoral response is composed of non-neutralizing and neutralizing antibodies targeting HIV-1 envelope glycoprotein, the only viral determinant exposed at the surface of both the virion and the infected cell. Non-neutralizing antibodies develop as soon as 1 week following infection. Interestingly, non-neutralizing antibodies to HIV-1 Env V1:V2 loop significantly

correlate with protection. Non-neutralizing antibodies can indeed recruit effector cells through their Fc fragment, triggering antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular virus inhibition (ADCVI) involving the secretion of antiviral cytokines (Chanzu and Ondondo, 2014). Described as soon as 1985 (Weiss et al., 1985), autologous neutralizing antibodies (NAbs) appear after several months of infection and cross-reactive antibodies develop 2 to 4 years later, as shown in Figure 4. However, both high sequence divergence and glycosylations constitute hindrances to the potency of NAbs. HIV-1 Env indeed carries a “Glycan shield” that restricts antibodies access to conserved receptor binding sites (see II.2.c. HIV-1 Env glycoprotein). In addition, as carbohydrate moieties appear as self to the immune system, the heavily glycosylated face of HIV-1 Env reduces the antigenicity of a large region on the gp120 surface called the “immunologically silent face” (Wyatt and Sodroski, 1998). Nevertheless, rare seropositive individuals are able to produce potent broadly Neutralizing Antibodies (bNAbs) capable of neutralizing most of HIV-1 strains. Mapping and structural definition of HIV-1 bNAbs epitopes has led important insights into how the humoral system naturally combats HIV-1 infection (for review see (Mouquet, 2014)), opening new avenues for therapeutical and vaccine design.

#### d. Examples of (partial) control of the infection

The alarming prognosis of HIV-1 disease progression is however counterbalanced by few examples of control of HIV-1 infection.

##### *i. Resistance to HIV-1 infection*

A 32bp deletion in the gene encoding HIV-1 co-receptor CCR5, called the *CCR5-Δ32* polymorphism, introduces a premature stop codon in the *CCR5* gene. Individuals homozygous for this deletion display abrogated CCR5 surface expression and are therefore resistant to R5-tropic HIV-1 viruses that account for the majority of transmitted HIV-1 strains (Liu et al., 1996). In 2008, an HIV-1 patient suffering from leukemia underwent hematopoietic stem cells transplantations from a *CCR5Δ32* homozygous donor. After 6 years without HAART, this patient, now known as the Berlin patient, still has no detectable HIV (Allers et al., 2011), highlighting the importance of coreceptors in initiating and maintaining HIV infection.



## ***ii. Long Term Non Progressors and Elite controllers***

Long Term Non Progressors (LTNPs) represent 2 to 5 % of HIV-seropositive individuals. Their clinical picture is characterized by the absence of progression to AIDS for 10-20 years in the absence of therapy, associated to elevated CD4+ T-cell counts > 500 cells/uL. LTNPs are sometimes referred to as viremic controllers, as their plasmatic viral load is usually comprised between 50 and 2,000 copies/mL.

Among LTNPs, 0.55 % are considered as Elite Controllers (ECs) or elite suppressors as they maintain undetectable levels of plasmatic viral RNA (<50-75 copies/mL) (Poropatich and Sullivan, 2011).

In addition, some patients called Post-treatment Controllers (PTC) display a control of HIV-1 infection for several years after having stopped early-initiated HAART (cf French VISCONTI cohort of PTCs (Saez-Cirion et al., 2013)).

The precise mechanism of viral control in LTNPs, ECs and PTCs is unknown and may be different from one individual to another. Above all, this mechanism may be multifactorial and include viral, genetic and immunological aspects. Some studies have shown that virus strains from LTNPs are less evolved and thus less capable of evading the host immunological response when compared with progressor strains (Poropatich and Sullivan, 2011). In parallel, the over-representation of certain MHC class 1 alleles in LTNPs and ECs, including class I HLA-B\*57 and HLA-B\*27 alleles, represents a genetic factor associated to viral control (Kaslow et al., 1996). In addition, some intrinsic cell resistance factors, including p21 in macrophages, participate in the control of HIV-1 viremia in controllers (Allers et al., 2011). Sustained innate host immune responses might constitute another mechanism by which virus is controlled in these individuals. Some LTNPs indeed display an enrichment in specific natural killer (NK) cell immunoglobulin-like receptors (KIR) alleles known to strongly inhibit HIV replication in target cells *in vitro* (Cockerham and Hatano, 2015). In addition, although the frequency of HIV specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) is not elevated in ECs, their CTLs seem to produce more cytolytic proteins, such as perforin and granzyme B, and more pro-inflammatory cytokines, including interleukin (IL-2) and interferon-gamma (IFN- $\gamma$ ). It is also possible that CTLs restricted to the protective HLA-B\*57 and HLA-B\*27 alleles are more potent and polyfunctional (Cockerham and Hatano, 2015).

Overall, LTNPs and ECs are likely a heterogeneous group with multiple potential mechanisms of control. This group of individuals provides a unique opportunity to better understand viral persistence and host control.

### *iii. Elite Neutralizers*

Elite Neutralizers represent less than 1 % of HIV-1 infected individuals. They are characterized by their ability to produce broadly Neutralizing Antibodies (bNAbs). bNAbs neutralize more than one pseudovirus at an IC<sub>50</sub> titer of 300 within a clade group and across at least four clade groups (Simek et al., 2009).

The next step forward is to determine whether these specific antibodies are found in LTNP and EC subjects and whether they directly correlate with reduced viral loads.

## **3. Current therapeutical strategies**

### **a. Antiretroviral Treatment (ArT)**

Thirty years after the approval of zidovudine (AZT) by the Food and Drug Administration of the United States (US FDA), viral and human genes involved in the regulation of a variety of viral and host functions have been extensively characterized. Consequently, research has rapidly utilized this knowledge to design drugs that block viral replication.

Theoretically, all of the steps of HIV-1 life cycle (described in I.3.4. Early phases of HIV-1 replication cycle) represent targets for anti-retroviral therapy (Teixeira et al., 2011). However, to date the two major groups of drugs being used in clinical practice are **reverse transcriptase inhibitors** (nucleoside/nucleotide, NRTI, and non-nucleoside, NNRTI) and **protease inhibitors** (PI).

In recent years, three additional drugs have been approved, each representing a new class of antiretroviral inhibitors by targeting different steps of HIV-1 life cycle: AMD3100 and Maraviroc, which target the HIV-1 **entry** co-factors CXCR4 and CCR5 respectively (Donzella et al., 1998; Fatkenheuer et al., 2005), Enfuvirtide, which binds to gp41 and blocks the **fusion** process (Robertson, 2003), and Raltegravir, which targets the viral **integrase** (Grinsztejn et al., 2007). Currently available molecules for anti-HIV-1 antiretroviral treatment (ArT) are described in Table 2.

**Table 2. Current antiretroviral treatments (ArT) available for AIDS therapy.***(From (Teixeira et al., 2011)).*

Step	Target	Drug class	Substance (abbreviation, commercial name)
<b>Entry</b>	CCR5	Entry inhibitor	Maraviroc (UK-427 857, Selzentry)
<b>Fusion</b>	gp41	Fusion inhibitor	Enfuvirtide (T20, Fuzeon)
<b>Integration</b>	IN	Integrase inhibitor	Raltegravir (MK-0518, Isenstress)
<b>Reverse transcription</b>	RT	NRTI	Abacavir (ABC, Ziagen <sup>®</sup> ), Didanosine (ddI, Videx <sup>®</sup> ), Emtricitabine (FTC, Emtriva <sup>®</sup> ), Stavudine (d4T, Zerit <sup>®</sup> ), Lamivudine (3TC, Epivir <sup>®</sup> ), Tenofovir (DF, Viread <sup>®</sup> ), Zalcitabine (ddC, Hivid <sup>®</sup> ), Zidovudine (AZT, Retrovir <sup>®</sup> )
<b>Reverse transcription</b>	RT	NNRTI	Delavirdine (DLV, Rescriptor <sup>®</sup> ), Efavirenz (EFV, Sustiva <sup>®</sup> ), Etravirine (TMC125, Intelence <sup>®</sup> ), Nevirapine (NVP, Viramune <sup>®</sup> ), Rilpivirine (Edurant)
<b>Maturation</b>	PR	Protease inhibitor	Amprenavir (AMP, Agenerase <sup>®</sup> ), Atazanavir (ATZ, Reyataz <sup>®</sup> ), Darunavir (TMC114, Prezista <sup>®</sup> ), Fosamprenavir (GW-433908, Lexiva <sup>®</sup> ), Indinavir (IDV, Crixivan <sup>®</sup> ), Lopinavir combined with Ritonavir (ABT-378, Kaletra <sup>®</sup> ), Nelfinavir (NFV, Viracept <sup>®</sup> ), Ritonavir (RTV, Norvir <sup>®</sup> ), Saquinavir (SQV, Fortovase <sup>®</sup> , Invirase <sup>®</sup> ), Tipranavir (TPV, Aptivus <sup>®</sup> )

Current standard treatment for HIV infection, Highly Active Antiretroviral Therapy (HAART) is initiated when CD4 cell count drops below 500. HAART consists in the use of two or more antiretroviral medications, one of which having to be a **PI**, one of the NRTIs abacavir (ABC) or tenofovir (DF), an NNRTI, an integrase inhibitor, or an entry inhibitor. Once-daily single-tablet regimens comprising a non-thymidine NRTI backbone (DF + FTC or DF + 3TC) and one NNRTI (EFV) are maintained as the preferred choices. In France, the 2013 « Morlat » report recommends 2 NRTI associated to a 3rd anti-HIV agent as first line treatment (Morlat, 2013). In most cases, when minor adverse effects are encountered, HAART is able to yield viral suppression i.e. HIV RNA copies < 50/mL as early as 2 to 4 weeks after initiation (Ripamonti et al., 2013).

However, one of the main obstacles in combating the global HIV epidemics is a rapid evolution of the virus by the selection of drug resistance mutations. Nowadays, mutations conferring resistance to virtually all the drugs on the market have hence emerged.

As a general mechanism of resistance, HIV often mutates in the targeted site of the viral protein, hampering the interaction with pharmaceutical inhibitors. For instance, mutations

across the envelope gene lead to resistance to entry inhibitors (De Feo and Weiss, 2012), mutations in the active site of PR induce resistance to PI (Lee et al., 2012). Mutations can even confer cross-resistance to different classes of inhibitors such as NRTI and NNRTI (Nikolenko et al., 2010). In addition, HIV-1 counterbalances potential increase in the fitness cost by compensating mutations (enhanced enzyme activity, increased sensitivity of the substrate cleavage site...) (Lee et al., 2012), making viral suppression even more difficult to reach.

The medical counter-acting strategy to the emergence of resistances consists in providing patients with second-line treatments. WHO recommends the use of two NRTIs in combination with a so-called “boosted PI” as second-line regimen (World Health Organization, 2013). This therapeutic strategy consists in providing a small dose of ritonavir concurrently with another PI to pharmacologically enhance exposure to the latter PI.

## b. Immune control as a therapeutical goal

### *i. The quest for an anti-HIV vaccine*

Soon after HIV-1 was isolated and confirmed as the cause of AIDS in 1983–1984, it was widely expected that an effective vaccine would be rapidly developed. Thirty years have passed and we are still struggling to develop an elusive vaccine.

Identification of the major structural proteins of the virus (Robey et al., 1985), cloning and sequencing of HIV genome (Wain-Hobson et al., 1985), description of neutralizing antibodies (Weiss et al., 1985) and establishment of non-human primate (NHP) models for HIV infection and vaccine development (Alter et al., 1984; Daniel et al., 1985) participated in the rapid initiation of the engineering of an envelope-based vaccine.

Initial vaccination strategies aimed at eliciting an anti-HIV-1 humoral response. targeting conserved conformational epitopes in the native envelope glycoprotein gp120. Taking into account the genetic variability of HIV-1 isolates and the use of different coreceptors, recombinant subunit vaccine AIDSVAX was hence designed as bivalent gp120 preparations derived from R5 and X4 strains and tested in two efficacy trials conducted from 1998 to 2003 in North America and Thailand. However, vaccines failed to confer protection against HIV, highlighting the limitations of antibody-based vaccines.

Vaccination strategies were therefore rapidly designed to trigger both humoral and cellular responses. Induction of CTL responses can be achieved through the use of live vectors that can simulate a natural infection and therefore efficiently present antigens to the immune system. Over the last 20 years, 71 trials have thus been conducted with pox-virus based HIV vaccines. For instance, ALVAC<sup>®</sup> was developed as a non-replicating Canarypox-based vaccine expressing *gag* and other HIV genes along with HIV-1 Env, with the aim of inducing a broad cell-mediated immune response. The RV144 study conducted from 2003 to 2009 in Thailand tested the safety and efficacy of a prime-boost regimen comprising an ALVAC-HIV prime followed by booster injections of the gp120 subunit vaccine AIDSVAX. RV144 achieved 31.2 % efficacy and therefore constitutes the only efficient anti-HIV vaccine trial ever conducted in humans (Rerks-Ngarm et al., 2009).

Ongoing research now focuses on the induction of long-term potent protection, by inducing preferential maturation of naïve B-cell precursors into long-lived antibody-secreting cells (ASC) using for instance adenoviruses encoding cytokines such as IL-4, IL-5 or IL-6 as genetic adjuvants. As dendritic cells (DCs) represent the most potent and the most effective APCs at activating naïve T cells, they greatly enhance both CTL and antibody responses. Recent vaccination strategies hence aim at targeting antigens to DCs by incorporating PAMPs that target DC-expressed PRRs, using particulate delivery systems that mimic the size and shape of pathogens or antibodies against DC-expressed cell-surface receptors (review see (Moyle and Toth, 2013).

In addition, glycans are no longer seen only as a shield but can represent vulnerability sites that should be considered for the design of vaccine immunogens (Mouquet, 2014). The use of engineered glycoproteins and other glycostructures as vaccines to elicit antibodies with broad neutralizing activity represents a key area of interest in HIV vaccine design. Isolated from an Elite Neutralizer, 2G12 binds a cluster of two to four high-mannose (Man<sub>8</sub>GlcNAc<sub>2</sub> and/or Man<sub>9</sub>GlcNAc<sub>2</sub>) glycans on HIV-1 Env gp120. On the basis of this information, many groups have worked to design multivalent oligomannose clusters that might mimic the 2G12 epitope for use as vaccines to stimulate a 2G12-like antibody response (Horiya et al., 2014).

## ***ii. Broadly Neutralizing Antibodies (bNAbs)***

Favoring the generation of broadly Neutralizing Antibodies appears as a seducing therapeutical option potentially conferring protection to a broad range of HIV-1 strains.

Recent vaccination strategies hence aim at triggering activation-induced cytidine deaminase (AID) that could potentially enhance the generation of bNAbs by favoring somatic hypermutation (Seidl et al., 2012). Long-term production of high titers of bNAbs can also be achieved using adeno-associated virus (AAV) encoding bNAbs, eliciting a protection not only against intravenous challenge (Balazs et al., 2012) but also in the case of mucosal exposure to the virus (Balazs et al., 2014). Similarly, reduction of viremia was recently achieved in HIV-1 infected patients through direct infusion of bNAb 3BNC117 (Caskey et al., 2015). However, passive administration of potent neutralizing human antibodies does not eliminate infection in hu-PBL-SCID mice, but instead selects for variants with mutations in multiple antigenic sites (Andrus et al., 1998). It is thus likely that eliciting a monoclonal antibody response in infected individuals may be inefficient at clearing the infection but rather drives viral diversification (Mosier, 2000). An emerging view thus suggests the use of a combination of bNAbs to enhance viral clearance (Horwitz et al., 2013; Seidl et al., 2012).

### *iii. New therapeutic strategies*

As the genital mucosa represents a major portal of entry for HIV-1, halving HIV-1 transmission for instance using genital microbicides is of key importance. Over the last years, several lectin-based microbicides targeting HIV-1 Env glycosylations have been developed and tested in preclinical studies (for review see (Huskens and Schols, 2012)). In addition, the mucosa constitutes the first line of immune defense against HIV-1 infection. A major avenue of research currently aims at inducing CTL and IgG/IgA responses at the genital mucosa.

Another field of research focuses on increasing the potential viral targets of ArT by developing new drugs. For instance, assembly inhibitors that interfere with the critical CAp24 NTD-CTD intermolecular interactions involved in the stabilization of HIV-1 fullerene core (see II.2.b. Structural features of HIV-1 viral particle) are currently developed and characterized (Lemke et al., 2012).

As HIV-1 cellular reservoirs (defined in II.3. Cellular targets and reservoirs) represent a major hindrance to complete viral clearance, reactivation from latency also appears as a valuable therapeutic option (Suyama et al., 2009). Along those lines, the “Shock-and-Kill” strategy was designed, consisting in inducing HIV-1 escape from latency in the presence of ArT. Reactivated virus-producing cells are thus made visible to ArT and immune cells for elimination. Stimulation of replication of latent virus in reservoir cells was recently successfully achieved by suberoylanilide hydroxamic acid (SAHA), an inhibitor of Histone

Deacetylases (HDAC) involved in down-regulation of gene expression (Shishido et al., 2012). Similarly, Sogaard *et al.* reported at the 20<sup>th</sup> international AIDS conference in July 2014 the reinduction of viral replication by the HDAC inhibitor Romidepsin in 6 patients undergoing ArT. Levels of cell-associated HIV RNA in CD4 cells as well as plasma HIV RNA rose significantly. Unfortunately, no reduction in the viral reservoir was observed, as measured by total HIV DNA in CD4 cells. These results seem to indicate the need for a vaccine to improve the efficiency of the Killing phase. Also presented at the 20<sup>th</sup> international AIDS conference, interesting alternative strategy propose to maintain proviruses in a permanent latent state, through the use of inhibitors of transcription such as mTOR.

## II. Human Immunodeficiency Virus type I, etiological agent for AIDS

### 1. Classification of HIV-1

HIV-1 belongs to the family of *Retroviridae*, subfamily *Orthoretrovirinae* (orthoretroviruses), genus *Lentivirus*.

According to the sequence of their Env gene, HIV-1 strains are divided into two major groups, the M (major) and the O (outlier) group, the M group representing as much as 90 % of HIV-1 infections worldwide. Two less represented groups N and P complete this classification, leading to 30 to 50 % intergroup sequence divergence. Groups are further divided into subtypes or clades that display an interclade sequence divergence of 20 to 30% and an intraclade divergence of 10 to 15 %. Thus, the M group is sub-classified into 9 clades, named A, B, C, D, F, G, H, J and K. Upon co-infection of the same target cell with virions belonging to two different clades, Circulating Recombinant Forms can arise. Clades A and C are the most widespread whereas clade B is the most represented in Europe and in the USA. As a consequence, most drugs are designed to target clade B (Spira et al., 2003). In this study, we will primarily focus on HIV-1 primary virus isolates and laboratory strains that belong to group M clade B.

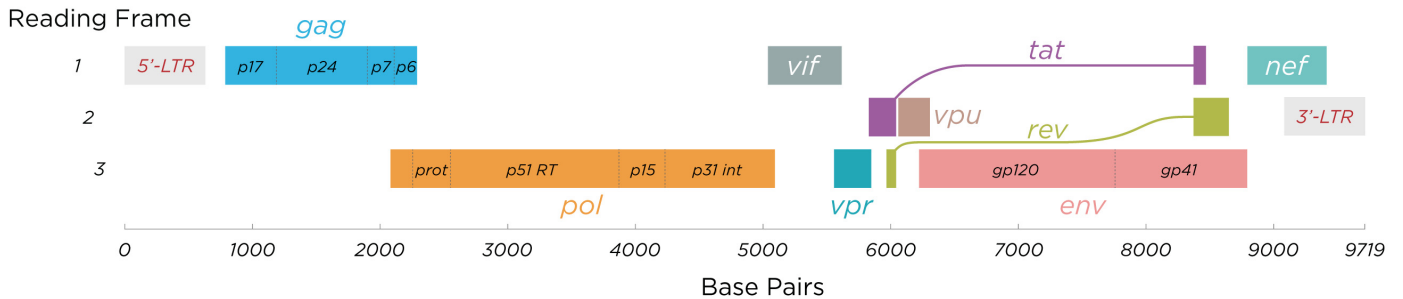
### 2. Organization of HIV-1 viral particle

#### a. Genomic organization of HIV-1

HIV-1 genome is composed of two copies of non-covalently linked, unspliced, positive-single-stranded RNA that are 9,749 nucleotides long. This RNA genome bears a 5' cap (Gppp) and a 3' poly(A) tail. It displays several structural landmarks such as the Long Terminal Repeats (LTR), Trans-activation Response element (TAR) and Rev Responsive Elements (RRE), and consists 9 Open Reading Frames (ORF): **gag**, **pol**, **env**, **tat**, **rev**, **nef**, **vif**, **vpr**, **vpu**, encoding 15 proteins as depicted in Figure 5. Though they may be altered by mutation, all of these genes exist in all known variants of HIV-1. HIV employs a sophisticated system of differential RNA splicing to obtain the Gag, Pol and Env polyprotein precursors as well as additional RNAs encoding HIV-1 auxiliary proteins. Gag precursor is later proteolytically processed to generate the matrix (MA), capsid (CA), nucleocapsid (NC), and



p6 proteins, the Gag-Pol polyprotein adds protease (PR), reverse transcriptase (RT), and integrase (IN) while the Env precursor yields Env gp120 and gp41 (for more detailed description see (Watts et al., 2009)).

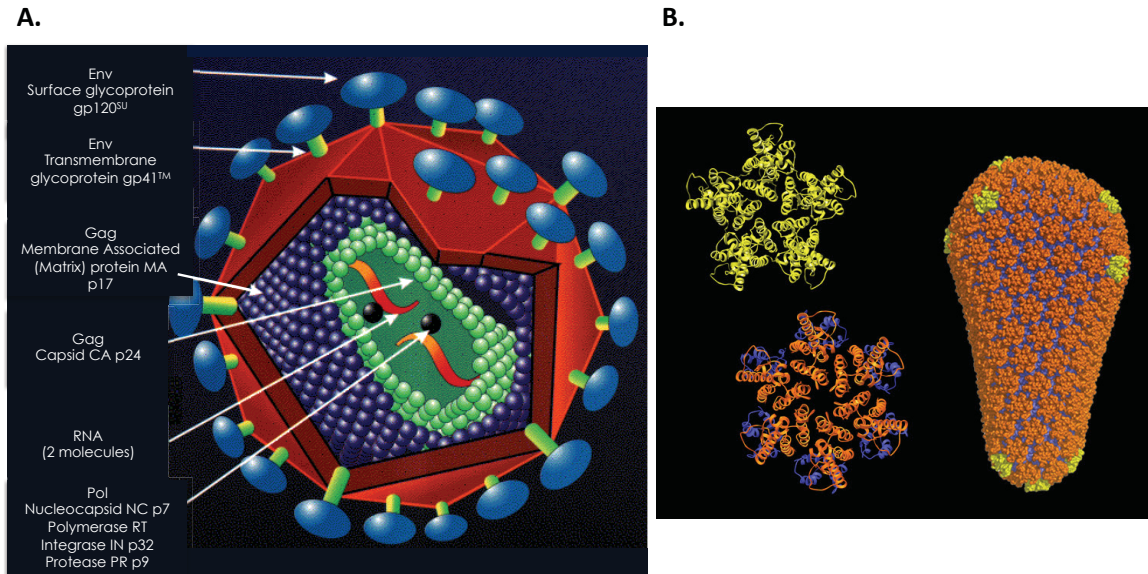


**Figure 5. Organization of HIV-1 genome.**

Scheme representing HIV-1 genome, composed of the 5' LTR, the 3' LTR and 9 ORFs : **gag**, encoding M<sub>Ap</sub>17, C<sub>Ap</sub>24, p6 and p7, **pol** encoding PR (marked prot), RT and INT, **env** encoding gp120<sup>SU</sup> and gp41<sup>TM</sup> as well as **Vif**, **Vpr**, **Vpu**, **Tat**, **Rev** and **Nef** (from <http://www.scistyle.com/>).

## b. Structural features of HIV-1 viral particle

HIV-1 is a roughly spherical large virus with a diameter of about 120 nm (range 90 to 160 nm), as measured on thin section by Transmission Electron Microscopy (TEM) (Reicin et al., 1996). As depicted in Figure 6A, mature HIV-1 viral particles contain two copies of a positive single-stranded RNA tightly bound to viral Nucleocapsid NC p7, Reverse Transcriptase RT, Integrase IN and Protease PR. This genetic material and its associated proteins are enclosed in a « core » composed of about 1,500 copies of the mature viral capsid protein C<sub>Ap</sub>24. This core is organized in a conical fullerene shell composed of an uninterrupted lattice of C<sub>Ap</sub>24 hexamers and pentamers, as shown in Figure 6B.



**Figure 6. Structure of mature HIV viral particle and of its capsid core.**

*A. HIV viral particle contains 2 RNA molecules associated to Pol proteins Nucleocapsid NC p7, Protease PR p9, Polymerase RT, Integrase IN p32. This complex of nucleic acid and proteins is enclosed in a conical capsid formed of capsid proteins CA p24. A layer of Membrane Associated protein MA p17 surrounds this conical capsid and interacts with a lipid bilayer that surrounds the core and forms an envelope of 120 nm in diameter. HIV envelope is bearing resident host transmembrane proteins as well as  $14 \pm 7$  trimeric Env spikes composed of a stem of three transmembrane envelope glycoproteins gp41<sup>TM</sup> capped by three surface envelope glycoprotein gp120<sup>SU</sup>. B. HIV capsid core is organized in a conical fullerene shell (right), composed of approximately 250 hexamers of mature CA p24 (orange and violet, bottom left), five CA p24 pentamers (yellow, upper left) at the narrow end and seven at the wide end.*

A matrix composed of the viral capsid protein MAp17 surrounds the core and closely interacts with the viral envelope, a phospholipid bilayer originating from the membrane of the host cell and containing spikes of the viral Env protein.

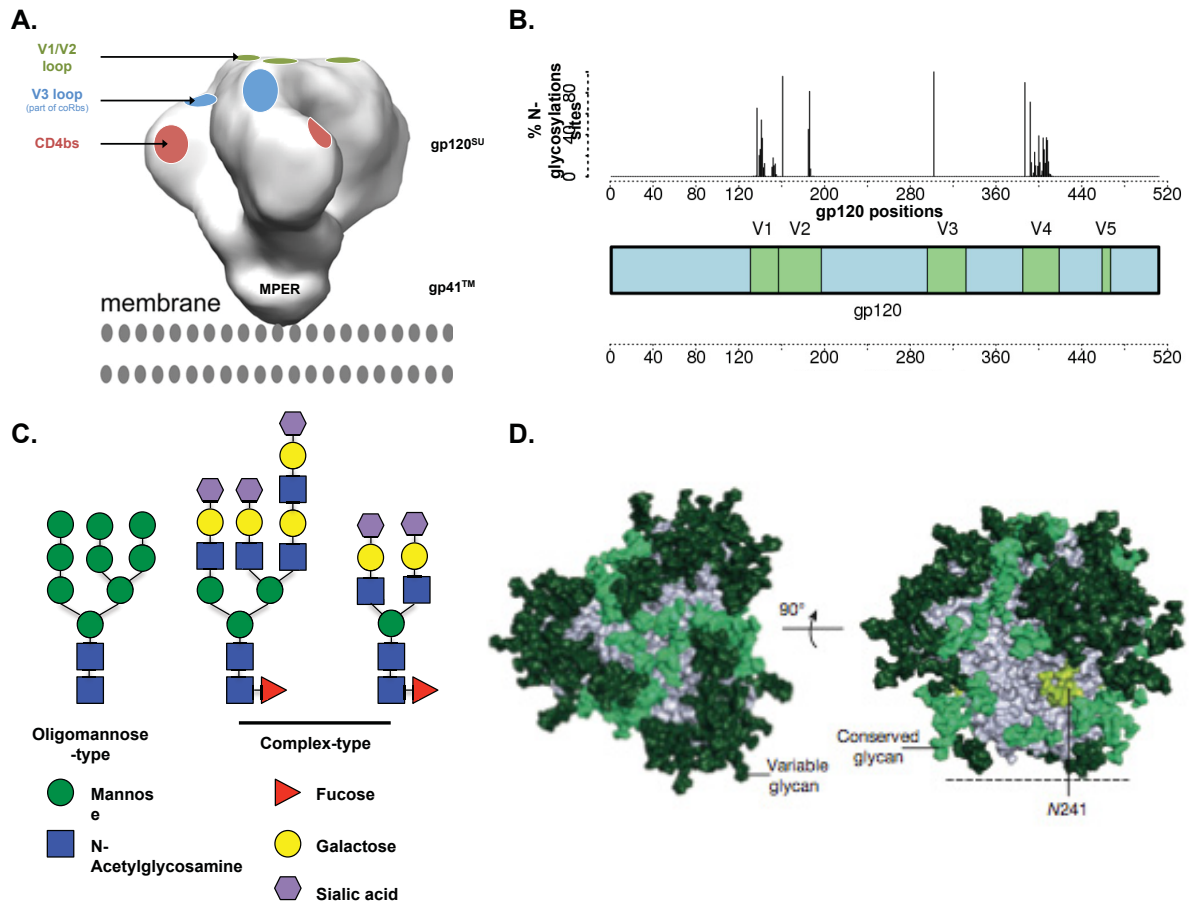
### c. HIV-1 Env glycoprotein

Env spikes are trimeric structures (Weissenhorn et al., 1997) synthesized as a precursor, gp160, which oligomerizes upon folding within the endoplasmic reticulum (ER). Once shuttled to the Golgi apparatus, Env precursor spikes are proteolytically cleaved by host proteases into a cap made of three surface envelope glycoproteins (gp120<sup>SU</sup>) and a stem composed of three transmembrane envelope glycoproteins (gp41<sup>TM</sup>), which anchor the spike in the viral lipid bilayer through the Membrane Proximal External Region (MPER). As depicted in Figure 7.A, each monomer of gp120<sup>SU</sup> harbors a CD4 binding site (CD4bs), a

V1/V2 loop and a V3 loop that participates in the coreceptor binding site (coRbs) and dictates coreceptor usage (described in II.3. Cellular targets and reservoirs).

Each HIV-1 viral particle carries  $14 \pm 7$  (range 4 to 35) spikes of viral Env protein, as measured by cryoEM analysis of wild-type HIV-1 virions (Zhu et al., 2006). This number is relatively low as compared to other retroviruses (Schiller and Chackerian, 2014). In addition, for some HIV-1 strains, and especially the X4-tropic clone HXB3, a rather rapid ( $t_{1/2} = 57$  h) and spontaneous shedding of HIV-1 gp120 leads to a further decrease in the number of spikes, contributing to a rapid decay in cell-free infectivity (Layne et al., 1992). However, although reported by others (Schneider et al., 1986), shedding of HIV-1 gp120<sup>SU</sup> does not seem to constitute a common feature for all HIV-1 or SIV isolates (Chertova et al., 2002).

Although no O-linked glycosylations (bound to serine/threonine) were reported, HIV-1 Env sequence harbors 18-33 (median 25) putative N-linked glycosylation sites (bound to asparagine) (Korber et al., 2001). The glycosylation pattern of HIV-1 Env is hence very rich, representing about 50 % of gp120 molecular mass. Many of these glycosylation sites are preserved in different HIV isolates even though some are found in hypervariable regions of gp120 as shown in Figure 7. HIV-1 gp120 carbohydrate moieties are enriched in oligomannosidic and complex-type glycans. Almost half of Env glycans have terminal mannose residues, mainly composed of Man9-Man7 species. Hybrid glycans comprise fucosylated and partially sialylated bi-, tri-, and tetraantennary oligosaccharide species as shown in Figure 7.B (Geyer et al., 1988). This glycan canopy, called the “glycan shield”, acts as a steric hindrance preventing NAbs from binding neighbouring epitopes, including those at V1/V2, V3, and the receptor binding surfaces (Wei et al., 2003).



**Figure 7. Structure and glycosylations of HIV-1 Env trimer.**

*A.* HIV-1 Env trimer density map obtained from cryo-EM shows the interaction of gp41<sup>TM</sup> Membrane Proximal External Region (MPER) with the lipid bilayer of the virion, noted membrane. Gp41<sup>TM</sup> is capped by a trimer of gp120<sup>SU</sup> displaying per monomer: 1 CD4 binding sites (CD4bs in red), as well as 1 V1/V2 loop at the top of the monomer and 1 V3 loops participating in the co-receptor binding site (coRbs). *B.* Mapping of the N-glycosylations and their respective frequencies on an alignment of all HIV-1 Env sequences reported in 2013 (www.hiv.lanl.gov), using HIV N-linked glycosylation site analyzer (Shaw and Zhang, 2013). *C.* Schematic representation of oligomannose-type and complex-type glycosylations carried by HIV-1 Env (From (Sato et al., 2012)). *D.* 3D representation of HIV-1 glycan shield, top view (left) and side view with the virion lipid bilayer at the bottom (right) display both conserved (dark green) and variable (light green) glycans at the surface of HIV-1 Env (From (Pancera et al., 2014)).

### 3. Cellular targets and reservoirs

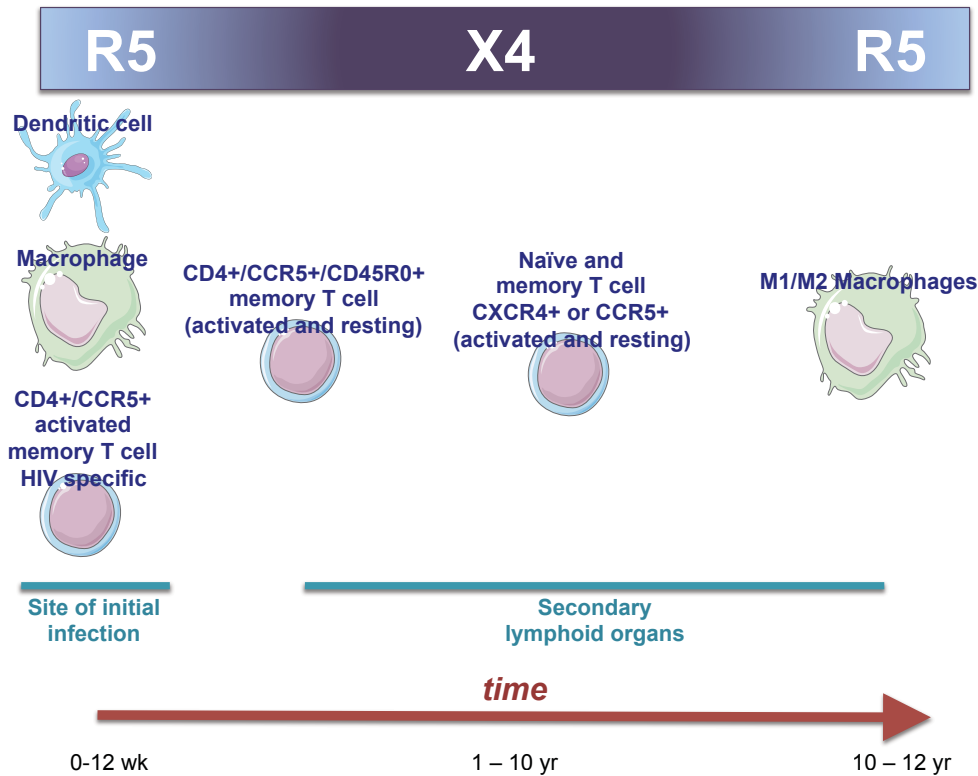
Obligate intracellular pathogen as any other virus, HIV primarily infects cells that express CD4 and one of the co-receptors CCR5 or CXCR4. Therefore, HIV targets a variety of immune cells such as macrophages, microglial cells, dendritic cells, and lymphocyte CD4+ T cells, which make up a quarter of the white blood cell count. However, some studies show that HIV-1 would also have the ability to infect cells lacking CD4, including B lymphocytes, astrocytes, thymocytes, CD8+ T lymphocytes, trophoblasts (Teixeira et al., 2011) and epithelial cells (Delezay et al., 1997)

At the inception of the infection, most sexually transmitted HIV-1 isolates use CCR5 as a coreceptor (Connor et al., 1997). As they continuously patrol the mucosal surface, macrophages, dendritic cells (DC) and CD4+/CCR5+ memory T cells are the first immune cells facing the virus and a significant proportion of macrophages of the lamina propria are susceptible to productive HIV-1 infection (Shen et al., 2009). Infected macrophages then efficiently transmit the infection to CD4+ T cells (Groot et al., 2008) and replication at the site of infection soon occurs mainly within CD4+ T cells (Zhang et al., 1999). Replication then quickly shifts to secondary lymphoid organs, especially lymph nodes, spleen, liver, bone marrow and gut associated lymphoid tissue (GALT) where HIV targets HIV-specific activated and resting memory CD45RO<sup>+</sup> CD4<sup>+</sup> T cells expressing both CD4 and CCR5 (Douek et al., 2002; Helbert et al., 1997) as depicted in Figure 8.

After years of infection, HIV-1 tropism evolves and the envelope sequences that determine coreceptor use mutate so that X4 viruses (using CXCR4 rather than CCR5 as a coreceptor) emerge as a detectable population in at least half of infected individuals (Mosier, 2000). Viruses either completely switch coreceptor from CCR5 to CXCR4 or sometimes retain their ability to use CCR5, becoming so-called dual-tropic viruses such as virus primary isolates DH12 (Shibata et al., 1995) and W132 (isolated by the group of Dr Asier Saez-Cirion). X4 HIV-1 variants predominantly infecting naïve resting CD4+ T cells. This change in coreceptor use thus expands HIV target cell population from memory CD4+ T cells to naïve (resting or activated) CD4+ T cells (Bleul et al., 1997) as well as CD62L+ memory CD4+ T cells (Gondois-Rey et al., 2002). Expansion in target cells availability as well as increase cytopathogenicity of X4 virus possibly results in a drastic acceleration of CD4 T cell loss (Bozzette et al., 1993). At later stages of the disease, when an accelerated depletion of CD4+ T cells by apoptosis occurs, HIV mainly buds from M1 classically activated

macrophages, reservoirs formed early in the course of the disease, and from alternatively activated M2 macrophages (Herbein and Varin, 2010).

Overall, CD4<sup>+</sup> T Lymphocytes represent the major replicating site for HIV-1, a feature responsible for the rapid drop observed in CD4 count along the course of the disease (see I.2.b. Symptoms and stages of the disease).



**Figure 8. Evolution of HIV cellular targets over the course of the infection.**

*At onset of the infection, HIV uses CCR5 as a coreceptor and targets tissue-resident dendritic cells and macrophages as well as activated memory HIV-specific CD4<sup>+</sup> CCR5<sup>+</sup> T cells. After a series of mutations in the viral envelope gene, HIV acquires the ability to use CXCR4 as a coreceptor, expanding its population of target cells to naïve activated and resting CCR5 and CXCR4 cells. At later stages of the disease, HIV is mainly produced by M1 and M2 activated macrophages (from (Mosier, 2000)).*

A major issue in the fight against HIV infection is the establishment of viral cellular reservoirs. Even if efficient ArT yields undetectable viral load, to date no therapeutical strategy has proven efficient at completely clearing HIV infection from the organism. HIV-1 infection is hence able to progress even though only a minute fraction of macrophages and resting/activated T cells is infected (Chun et al., 1997).

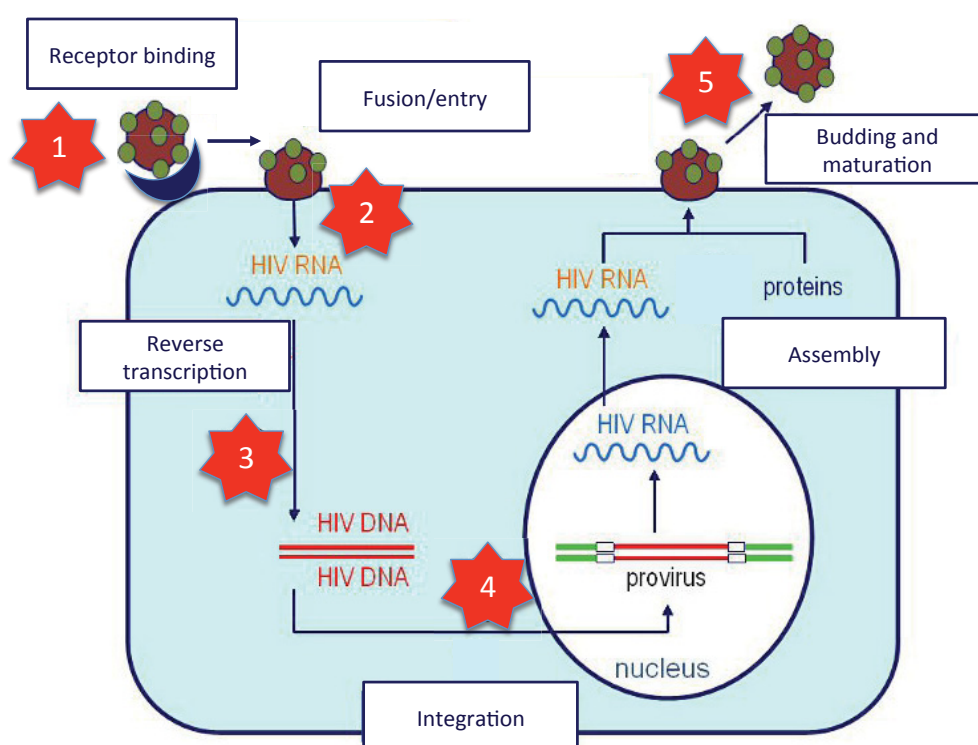
Early integration of HIV-1 provirus in T cells upon primary infection establishes resting T cells as a pool of latently infected cells (Chun et al., 1998) that poses challenges to eradicating infection (Zhang et al., 1999). As HIV-1 infection triggers a continuous low activation of the immune system, latently infected resting T cells can get activated, leading to the reactivation from latency of integrated HIV proviruses and *de novo* production of virions. Although circulating infected T cells survive only for an average 2.2 days (Perelson et al., 1996), T cell activation has been shown to favor AIDS progression (Kornfeld et al., 2005). In addition, follicular helper T cells from germinal centers also represent a major HIV-1 reservoir (for review see (Vinuesa, 2012)).

As HIV-1 infected macrophages are able to survive for extended periods of time (Koppensteiner et al., 2012), latently infected macrophages are considered as an important reservoir of HIV-1 infection that becomes more and more explored. The role of infected DCs as potential reservoirs in vivo remains controversial (Wu and KewalRamani, 2006).

While cellular reservoirs for HIV-1 latency are getting better characterized, remote anatomical sites harboring HIV-1 latency (Svicher et al., 2014) are imperfectly detected and eradication of HIV-1 reservoirs remains so far inaccessible.

## 4. Early phases of HIV-1 replication cycle

Regardless the target cell considered, HIV-1 viral cycle can be divided into early and late phases, described in Figure 9. Early phases of HIV-1 life cycle encompass all stages from viral particle entry to the target cell to the expression of the proviral genome. More specifically, HIV-1 entry into a host cell may be divided into 3 distinct steps: attachment, co-receptor interaction and fusion.



**Figure 9. HIV viral life cycle.**

**Early stages:** Using their envelope glycoprotein  $gp120^{SU}$ , HIV viral particles first bind their CD4 receptor at the plasma membrane of permissive cells. Viral  $gp120$  then interacts with the co-receptors CXCR4 or CCR5. Interaction with the co-receptors leads to a conformational change in the viral transmembrane glycoprotein  $gp41$ , causing the fusion of the virion with the plasma membrane of the host cell. After uncoating of the viral material, viral positive RNA undergoes reverse transcription to give rise to a dsDNA. Viral integrase  $IN$  then enables the integration of proviral dsDNA into the host cell genome.

**Late stages:** After translation and transcription of viral genomic material, viral proteins are shuttled to the plasma membrane where they assemble into viral particles. This assembly leads to the budding of viral particles from the plasma membrane, followed by a maturation step catalyzed by viral proteases.

Red stars recall steps currently amenable for therapeutical intervention.



### ***i. Viral particle attachment to the recipient cell***

Attachment of HIV-1 to target cells is mainly mediated by the interaction of HIV-1 Env with its receptor CD4 at the plasma membrane of a permissive cell. This gp120<sup>SU</sup>-CD4 complex interacts with a co-receptor on the cell surface, typically chemokine receptors CXCR4 or CCR5.

However, frequency of successful virus-cell interactions is considered rather low (Thomas et al., 2007) and other cellular molecules are cooperating with CD4 to increase the efficiency of HIV-1 attachment. Heparan sulfates proteoglycans (HSPGs) expressed at the surface of recipient cells hence increase HIV-1 attachment through interaction of their polyanionic sulfated side chains with basic residues within HIV-1 Env (Roderiquez et al., 1995). In addition, incorporation in HIV-1 virions of host transmembrane proteins such as ICAM1 (Fortin et al., 1998) and CD62L (Thibault et al., 2007) greatly enhances attachment to target cells (for incorporation of host proteins within HIV-1 viral particles, see II.4. Host proteins incorporated in HIV-1 particles upon budding).

Overall, these attachment factors favor HIV-1 Env/receptor-coreceptor binding, inducing conformational changes in the envelope glycoprotein that lead to the exposure of transmembrane glycoprotein (gp41<sup>TM</sup>) fusion peptide.

### ***ii. Viral fusion and capsid uncoating***

Subsequent to its conformational change, gp41<sup>TM</sup> then mediates pH-independent fusion of viral lipid bilayer with the membrane of the recipient cell. Fusion generally occurs at the plasma membrane of the target cells (Stein et al., 1987), although some studies indicate that productive infection can also occur through the endocytic pathway (Dale et al., 2011; Miyauchi et al., 2009). It was long believed that immediately after fusion, the capsid core is disrupted, enabling the release of capsid genomic and proteic contents into the cytoplasm of the host cell. However, it appears that uncoating rather occurs at the nuclear pore (Arhel et al., 2007) and capsid uncoating might be synchronized with HIV-1 nuclear entry (Di Nunzio, 2013).

### ***iii. Reverse transcription of the viral genomic material***

Reverse transcription of HIV-1 RNA is likely to occur within intact capsid cores (Arhel et al., 2007; Di Nunzio, 2013). Viral positive-strand RNA template is reverse-transcribed into its complementary DNA strand by the viral Reverse Transcriptase (RT). Unable to catalyze

the proofreading usually performed by a regular DNA polymerase, HIV-1 RT is highly error-prone. HIV-1 thus displays a high mutation rate of  $\sim 0.2$  errors per genome during each replication cycle. In addition, HIV-1 RT displays recombinogenic properties involving “jumps” between the two RNA templates during reverse transcription. Both these mechanisms favor the appearance of resistant strains (Onafuwa-Nuga and Telesnitsky, 2009; Rambaut et al., 2004). Following reverse transcription, RT degrades the RNA template and subsequently produces double-stranded viral DNA.

#### ***iv. Integration of the proviral DNA***

Newly synthesized viral dsDNA is then imported into the nucleus of the host cell. Viral DNA is processed and transferred to the host genome by the viral enzyme Integrase (IN). Once the viral DNA has been inserted, the virus is called a provirus and infection in that cell is for all intents and purposes permanent. Thereafter, HIV-1 can persist in a latent, proviral state for many years in unstimulated cells (for recent review see (Battistini and Sgarbanti, 2014)). Activation of the host cell leads to the expression and synthesis of the viral genome, enabling the production of new infectious viral particles.

#### ***v. Expression of HIV-1 proviral genome***

Together with the *cis*-acting RNA enhancer element TAR, HIV-1 accessory protein Tat is the major regulator of HIV-1 genomic expression and increases the efficiency of transcription of HIV-1 proviral DNA (Laspia et al., 1989). To enhance LTR activation, HIV-1 Tat interacts with cellular transcription factors including NF- $\kappa$ B, SP-1 (Karn and Stoltzfus, 2012) and C/EBP $\beta$  (Mameli et al., 2007). As a consequence, Tat also exerts its effects on the expression of host genes, leading to cellular dysregulation.

After transcription of proviral DNA by the host cell RNA polymerase, viral mRNA is exported outside the nucleus in a Rev-regulated manner (for review see (Vercruyssen and Daelemans, 2013)) and translated by ribosomes as any cellular mRNA. Viral RNA encodes viral capsid proteins, envelope proteins and other auxiliary proteins in several frames, requiring alternative splicing of the mRNA and use of alternative initiation sites for translation as shown in Figure 5. Translation and synthesis of viral proteins occurs in the rough endoplasmic reticulum, and proteins are then exported through the Golgi apparatus to the cell surface, conveyed by a complex machinery of cellular export proteins. Ultimately,

virus particle production involves a multistep process that includes: 1) Gag targeting to the site of virus assembly, 2) binding of Gag to lipid membrane bilayer, 3) Gag multimerization, and 4) budding and release of nascent virus particles. These tightly regulated steps are detailed in chapter III. Focus on the late stages of HIV-1 viral cycle.

### III. Focus on the late stages of HIV-1 viral cycle

#### 1. Sites of budding

HIV-1 has two major targets *in vivo*: macrophages, which constitute a long-lived HIV-1 reservoir, and T lymphocytes, that represent the major site of HIV-1 replication *in vivo*.

In macrophages viral particles assemble and are stored within intracellular compartments termed Virus-Containing Compartments (VCCs). Budding at the membrane of those internal compartments is similar to the biogenesis of exosomes in Multivesicular Bodies (MVBs) (Raposo et al., 2002). These shared location and mechanism of budding lead to a similar incorporation of host proteins for macrophage-derived exosomes and virions, leading to enrichment in MHC II, CD63 and tetraspanins (Chertova et al., 2006; Raposo et al., 2002). As a minor proportion of VCCs remains connected to the plasma membrane overtime (Gaudin et al., 2013), the majority of those virus-producing intracellular compartments transiently connect to the plasma membrane via microchannels (Bennett et al., 2009; Gaudin et al., 2013) Similarly, HeLa or Hek cell lines often used for production of viral stocks display a viral budding within MVBs (Sherer et al., 2003).

In CD4<sup>+</sup> T lymphocytes, HIV-1 assembly occurs at the plasma membrane. Myristoylations within the globular head of MAp17 indeed target Gag assembly to phosphoinositides PI(4,5)P<sub>2</sub> resident in the plasma membrane (Saad et al., 2006). More precisely, and similar to MLV (Li et al., 2014), HIV-1 assembly was shown to occur at the uropod of T cells (Llewellyn et al., 2010). The uropod constitutes a protrusion at the rear end of a migrating T cell, which is enriched in CD44 and intercellular adhesion molecules (del Pozo et al., 1995) as well as lipid rafts and tetraspanins. Specifically, lipid rafts have been proposed as preferred sites of budding for HIV-1 in T cells (Ono and Freed, 2001), leading to and enrichment of HIV-1 lipid bilayer in raft-specific proteins (Nguyen and Hildreth, 2000) and raft lipids such as cholesterol and sphingolipids (Brugger et al., 2006). In parallel, tetraspanin-enriched microdomains (TEMs) were shown to participate in HIV-1 assembly (Nydegger et al., 2006).

## 2. Assembly, budding and cellular partners involved

Gag addressing to the plasma membrane represents the initial step in viral particle assembly and shuttling of this major viral structural protein appears to be driven by cellular factors involved in membrane and vesicular trafficking.

Although no active transport of Gag seems required for particle assembly (Jouvenet et al., 2006), microtubule-associated motor protein KIF4 has been reported to bind and enable HIV-1 Gag assembly (Martinez et al., 2008) and KIF3A contributes to VCC export in macrophages (Gaudin et al., 2012).

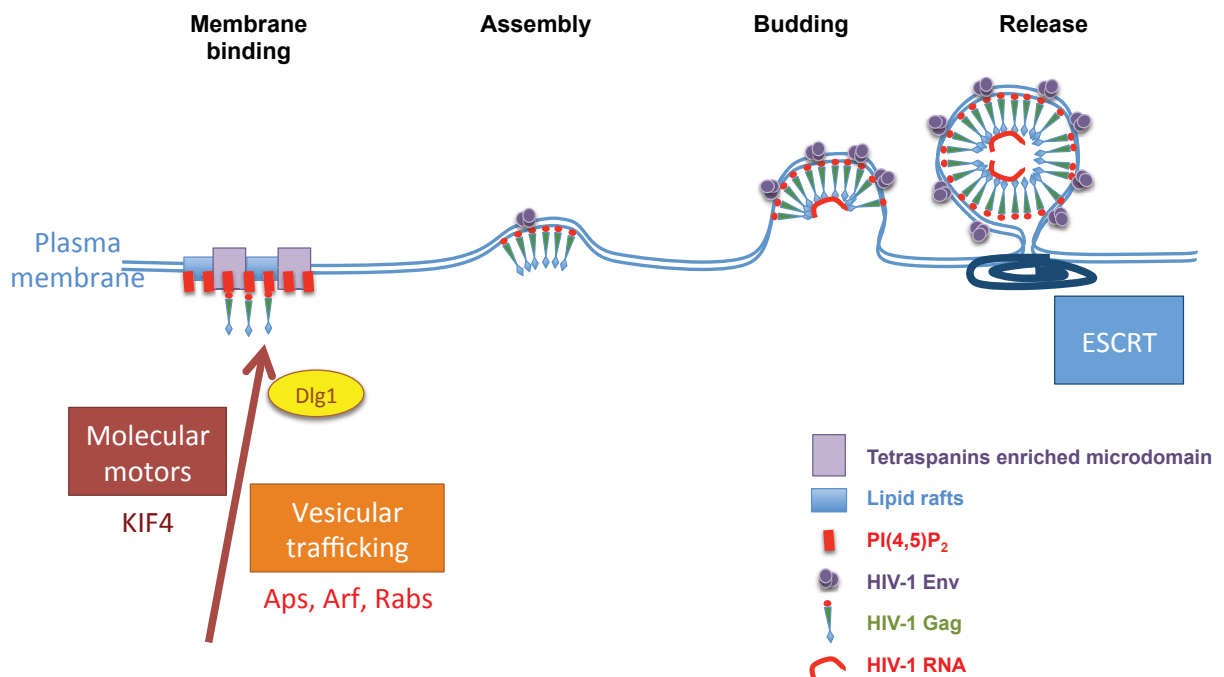
In addition, factors involved in endosomal trafficking and recycling have been reported to affect HIV-1 Gag localization. For example, HIV-1 Gag interacts with the adaptor complexes AP-1 (Camus et al., 2007), AP-2 (Batonick et al., 2005), and AP-3 (Dong et al., 2005) as well as GGA and Arf proteins (Joshi et al., 2008) that normally function as clathrin-adaptor proteins in vesicular trafficking. Some evidence further supports the subversion of endosomal trafficking for HIV-1 assembly, as late endosome-associated proteins Rab7A (Caillet et al., 2011) and Rab9 (Murray et al., 2005) are implicated in Gag trafficking. Interestingly, and relevant to this thesis, ECM proteins are conveyed to the extracellular milieu through similar endosomal secretory routes (Sung et al., 2011).

Subsequent to its proper trafficking to membrane microdomains, HIV-1 Gag is clustering with two copies of the viral genomic RNA under the plasma membrane of infected cells in so-called budding platforms (Jouvenet et al., 2008). Interactions between the matrix (MA) domain of Gag and the cytoplasmic tail of Env drive the incorporation of viral envelope glycoprotein Env, a critical step for the production of infectious viral particles (for review see (Tedbury and Freed, 2014)).

Viral particle assembly induces membrane curvature that allows virions to bud off the plasma membrane. The ESCRT pathway then catalyzes the severing of the membranous neck bridging the nascent viral particle to the cytoplasm. HIV-1 Gag indeed contains late-budding or 'L'-domains that bind directly or indirectly to components of the ESCRT pathway. The p6 domain of HIV-1 Gag hence binds Tsg101 (a component of ESCRT-I) and ALIX (an ESCRT-I and ESCRT-III binding protein), initiating the release of budding viral particles (for review see (Bieniasz, 2009)).

After budding, viral particles are either released as cell-free virions or remain associated to the cells. In macrophages, viral particles are indeed retained in VCC transiently connecting to the plasma membrane (Gaudin et al., 2013) while in T cells, HIV-1 virions can be retained

at the plasma membrane through IFN-inducible host protein Tetherin (Hammonds et al., 2010). While tetherin bridges viral particles together and to the plasma membrane (Perez-Caballero et al., 2009; Sauter et al., 2010), viral protein Vpu antagonizes this retention (Neil et al., 2008), ultimately favoring HIV-1 release as cell-free virions.



**Figure 10. Scheme of HIV-1 assembly and budding.**

*Conveyed by cellular molecular motors and molecules involved in vesicular trafficking, HIV-1 Gag is targeted to membrane microdomains enriched in lipid rafts and tetraspanins, and specifically binds PI(4,5)P<sub>2</sub>. HIV-1 Gag and Env then cluster together with HIV-1 RNA and induce membrane curvature. The ESCRT machinery severs viral particles and thus enables their release from the plasma membrane.*

### 3. Maturation

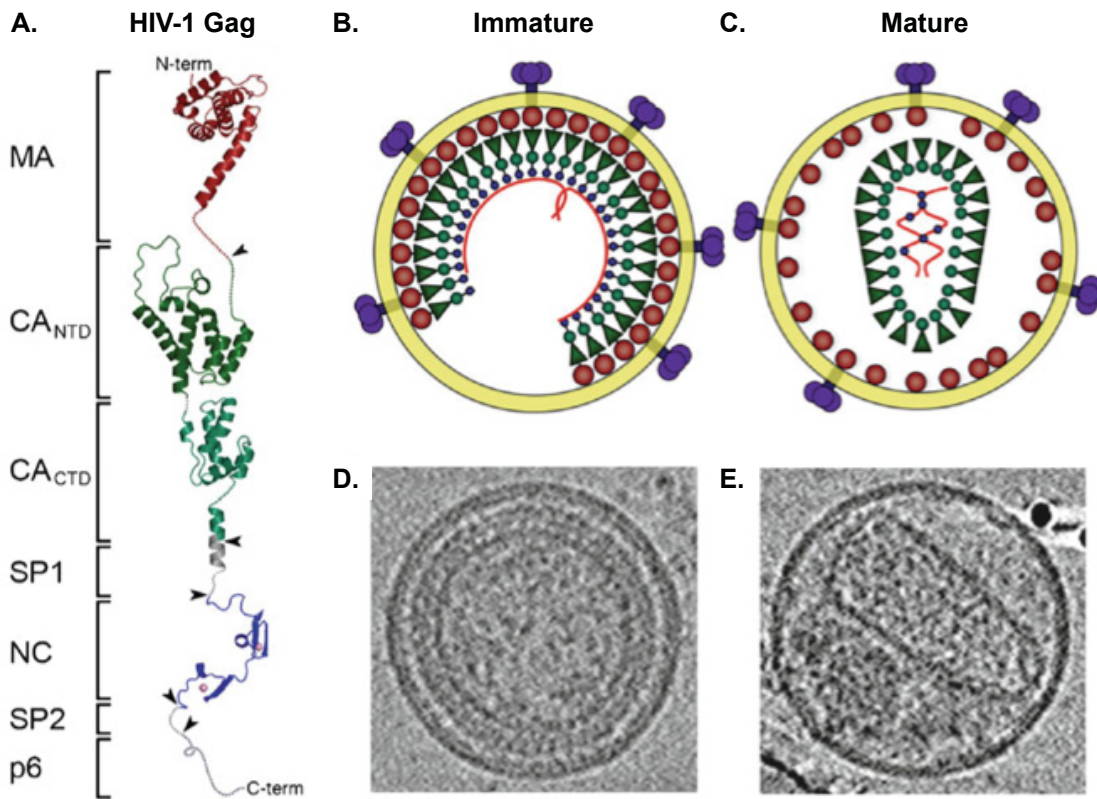
Upon budding from the plasma membrane of the host cell, the emergent virion is non infectious or immature and is characterized by a protein shell located immediately beneath the envelope. This protein shell is composed of ~2,400 Gag molecules embedded in the inner bilayer leaflet of the viral membrane via their N-terminal matrix (MA) protein as shown in

Figure 11A, with a crude estimate that Gag molecules occupy 50–60 % of the volume of the sphere (Lee, Potempa et al. 2012). Those immature virions also contain ~120 Gag-Pro-Pol molecules and yield a crescent shape in TEM as depicted in Figure 1 and Figure 11D.

Upon maturation, the embedded aspartic protease PR dimerizes, releases itself from the Gag-Pro-Pol precursor and cleaves this precursor into three new proteins: MA p17, CA p24, and NC p6 as shown in Figure 11A. From these cleaved products, NC condenses with the viral RNA and its associated enzymes into a compact ribonucleoproteic complex, MA forms the matrix layer associated with the viral lipid bilayer, and about 1,500 copies of the processed capsid protein CAp24 assemble into the fullerene conical shell displayed on Figure 6B and Figure 11E (Ganser-Pornillos et al., 2012), culminating in the formation of the mature, infectious virion depicted in Figure 11C&D. However, HIV-1 maturation aborts rather often, and virions harboring such a typical mature dense-core morphology make only up to 40% of NL4-3 culture supernatants (Ohishi et al., 2011). As maturation induces MAp17 release from its precursor, presence of the mature MAp17 protein constitutes a good marker of HIV maturation.

Although maturation has proven difficult to observe in the virion, it is clear that assembly, budding, and proteolytic processing of Gag and Gag-Pro-Pol are intricately linked events. Most textbooks indicate that processing occurs immediately after budding. However, it must be acknowledged that the timeframe of initiation of processing is unclear (Lee et al., 2012). Some studies even suggest that maturation could occur within endosomal compartments of the target cell (Dale et al., 2011; Miyauchi et al., 2009).

Maturation nevertheless appears as a crucial step determining viral particle infectivity, as proteolytic processing of HIV-1 Gag is linked to conformational changes within HIV-1 Env that lead to the acquisition of functional fusogenic properties (Joyner et al., 2011; Murakami et al., 2004).



**Figure 11. Relative organization and aspect of HIV immature and mature viral particles.**

*A. Ribbon representation of the structure of HIV-1 Gag precursor, displaying from N-Terminus (top) to C-Terminus (bottom): MA p17, CA p24 N-Terminal Domain CA<sub>NTD</sub>, CA p24 C-Terminal Domain CA<sub>CTD</sub>, Spacer Peptide 1 SP1, Nucleocapsid NC p9, Spacer Peptide 2 SP2 and p6. The same color code is conserved in B. and C.. B. Schematic representation of HIV immature viral particle, with a protein shell composed of ~2,400 Gag molecules embedded in the inner bilayer leaflet of the viral membrane via their N-terminal matrix (MA) domain. C. Schematic representation of HIV mature viral particle with MA proteins associated with the viral lipid bilayer, about 1,500 copies of the processed capsid protein CA p24 assembled into a fullerene conical core; and NC condensed with the viral RNA and its associated enzymes. D. and E. Transmission electron micrographs of immature and mature virions resp. preserved in vitreous ice. (From (Ganser-Pornillos et al., 2012).*



#### 4. Host proteins incorporated in HIV-1 particles upon budding

Since HIV-1 carries very few Env spikes, the numerous glycosylated proteins present on the virus surface are mainly host membrane components (Sato et al., 2012). While Moloney murine leukemia virus (MMLV) carries only eight host membrane proteins (Segura et al., 2008), HIV-1 indeed associates to and incorporates a broad variety of host proteins, ranging from nuclear and cytoplasmic proteins to membrane-associated proteins. The nature of incorporated host proteins strongly depends on the cell type used for viral production (Bastiani et al., 1997) in link with the cellular compartments and organelles that virions associate with during assembly, and by the relative abundance of particular proteins in these compartments (Iordanskiy et al., 2013). We will mainly focus on the incorporation of plasma membrane (PM) proteins into HIV-1 virions produced by infected T cells.

Cellular membrane proteins can be concentrated (sorted in), diluted (sorted out), or passively included into viral particles. Lipid-normalized protein comparison of Gag particles and donor membranes indicates that the bulk of host proteins in the donor membranes is mostly incorporated into HIV-1 particles in an undiluted fashion (Hammarstedt and Garoff, 2004).

However, some proteins in the donor membrane are excluded from the HIV-1 virions during budding. For instance, the integral membrane protein CD45 is present in microvesicles, yet does not appear to be incorporated into virions (Esser et al., 2001). This exclusion proves a useful tip for purification of viral particles from microvesicles (Chertova et al., 2006). Similarly, HLA-DQ, HLA-DP, and the HIV-1 receptor and coreceptors are not found in virions but are present at the PM. Exclusion of host proteins could result from the Pr55<sup>gag</sup> lattice functioning as a filter for transmembrane PM proteins with bulky cytoplasmic domains, such as CD45, or multiple transmembrane segments, such as the coreceptors. Alternatively, excluded proteins might form large oligomeric structures or associate with lipid microdomains that are left out of the budding particles (Hammarstedt and Garoff, 2004)

Conversely, a few proteins are concentrated into HIV-1 particles. HIV-1 preferential budding into lipid rafts in T cells indeed leads to the incorporation of raft-sequestered proteins such as Thy-1, GM1 and CD59 (Nguyen and Hildreth, 2000). In addition, Pr55<sup>gag</sup> appears as a driver for active incorporation of host proteins within HIV-1 lipid bilayer, as acidic residues

in the sequence encoding the C-terminal two-third of MA protein within Pr55<sup>gag</sup> polyprotein are crucial for the recruitment of ICAM-1 within HIV-1 viral particles (Jalaguier et al., 2015). In addition, HIV-1 virions seem to consistently incorporate the heperan sulfate adhesion protein CD44 (Bastiani et al., 1997; Bregnard et al., 2013; Chertova et al., 2006; Stephenson et al., 2010) as well as L-selectin CD62L (Bastiani et al., 1997; Thibault et al., 2007). Importantly, incorporation of CD44 and CD62L preserves their functionality and HIV-1 virions thus acquire the binding capacity of those incorporated host proteins (Guo and Hildreth, 1995; Thibault et al., 2007).

Overall, HIV-1 differentially incorporates a broad range of host proteins that might subsequently its HIV-1 infectivity, mostly through modified attachment to the target cell (Iordanskiy et al., 2013; Thibault et al., 2007).

## IV. HIV-1 cell-associated infectivity

### 1. Cell-free VS cell-associated infectivity *in vivo*

HIV-1 displays the ability to produce large amounts of cell-free viral particles, reaching an estimated average total HIV-1 production of  $10^{10}$  virions per day in an infected individual (Perelson et al., 1996). Cell-free viral particles can efficiently diffuse and spread HIV-1 infection, yet HIV-1 virions have a dramatically short life span of 0.3 days *in vivo* (Perelson et al., 1996). In addition, diffusion lowers HIV-1 plasmatic concentration, sometimes impairing the ability to isolate infectious viral particles from the serum (Michaelis and Levy, 1987).

As both cell-free virions and HIV-1 infected cells are detected in the body fluids vectoring HIV-1 transmission (Bagasra et al., 1994; Ceballos et al., 2009; Re et al., 1994; Van de Perre et al., 1992), HIV-1 infected cells are likely participating in HIV-1 propagation, from one individual to another but also within an infected individual, between and within organs. HIV-1 cellular targets T cells and macrophages have the ability to interact intimately with one another and with other cell types in physiological conditions. HIV-1 apparently diverts these cells to form junctions between these cell junctions to spread from one cell to another (Johnson and Huber, 2002).

At mucosal barriers, different cellular junctions support HIV-1 dissemination. HIV-1-infected cells are indeed able to form direct contacts with epithelial cells at the apical surface of mucosal tissue (Pearce-Pratt and Phillips, 1993), leading to efficient viral entry (Alfsen et al., 2005; Ganor et al., 2010). Epithelial cells then transcytose HIV-1 virions (Bomsel, 1997). Resident Langerhans cells (LCs) get rapidly infected following mucosal exposure to HIV-1 (Hu et al., 2000), either capturing transcytosed virions or projecting cellular processes through the epithelium to capture HIV-1 in the mucosa (Piguet and Sattentau, 2004). Ultimately, LCs can transmit a vigorous infection to CD4<sup>+</sup> T cells underneath the epithelial layer (Cameron et al., 1992; Fahrback et al., 2007).

In the blood of HIV-1 infected patients, about 1/100 to 1/1,000 CD4<sup>+</sup> T cell contains HIV-1 DNA, largely in the form of one integrated provirus (Josefsson et al., 2011). As part of their normal immune surveillance duties, CD4<sup>+</sup> T cells travel widely throughout the body and HIV-1 takes advantage of the migration of infected T cells to disseminate between secondary lymphoid organs (Murooka et al., 2012). Within secondary lymphoid organs, the primary site of HIV replication *in vivo*, HIV-1 infected cells intimately interact with neighboring non-infected cells (Ladinsky et al., 2014) and a high number of integrated proviruses (Jung et al.,

2002) as well as a compartmentalization of HIV-quasi species (Cheyner et al., 1994) are observed in the spleen of HIV-1 patients. HIV-1 dissemination within secondary lymphoid organs thus likely occurs in a cell-associated manner rather than *via* the diffusion of plasmatic cell-free virions. HIV hence takes advantage of physiological interactions between CD4<sup>+</sup> T cells and other effectors of the immune response to spread viral infection by cell-cell contact.

*In vitro* studies indeed demonstrate that HIV-1 efficiently spreads from one cell to another across junctions formed between T cells, dendritic cells (DCs) and macrophages. This cell-to-cell transfer involves either virological synapses formed between infected and target T cells (Jolly et al., 2004) or infectious synapses formed between DCs and T cells (Carr et al., 1999; McDonald et al., 2003; Tsunetsugu-Yokota et al., 1997), between macrophages and T cells (Duncan et al., 2013; Duncan et al., 2014; Groot et al., 2008), and even between infected T cells to macrophages (Baxter et al., 2014).

Overall, many cellular junctions enable HIV-1 cell-to-cell transfer. We will mainly focus on virological synapses formed between T cells, and on the *trans*-infection process that enhances HIV-1 transmission to T cells through the formation of infectious synapses between DCs and T cells.

## **2. Spotlight on two types of cellular junctions supporting HIV-1 cell-associated infectivity**

### **a. The virological synapse**

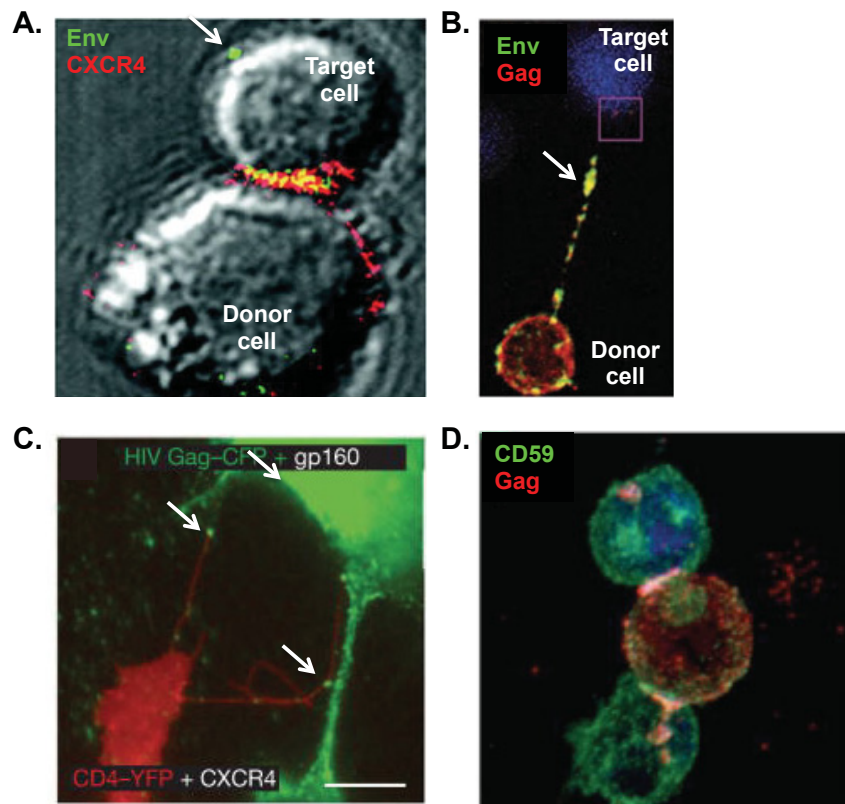
First described for the T-tropic virus Human T Cell Leukemia Virus Type I (HTLV-I), the virological synapse (VS) was characterized as a close and organized contact between an infected and a recipient cell enabling the transfer of viral material, possibly through cytoskeleton-mediated propulsion (Igakura et al., 2003). Soon thereafter, the paradigm of virological synapse was extended to HIV-1 transmission between T cells, as shown in Figure 13A (Jolly et al., 2004). Upon engagement of HIV-1 Env with CD4 and CXCR4 at the surface of the target cell, actin remodeling is induced, CD4, CXCR4 and HIV-1 Gag and Env are efficiently clustered and HIV-1 is transferred to the target cells (Jolly et al., 2004).

The initial description of the virological synapse made it strikingly reminiscent of the immunological synapse, a cellular junction establishing between an antigen-presenting cell and a T lymphocyte. As a consequence, many groups searched to characterize the

involvement of T cell molecular actors of the immune synapse in the formation of the virological synapse. Tetraspanins (CD63, CD81 and CD9) (Jolly and Sattentau, 2007) as well as adhesion molecules (LFA-1, ICAM-1, ICAM-3) (Jolly et al., 2004; Jolly et al., 2007) involved in IS formation, were thus shown to be recruited at the site of cell-cell contact at the VS. Similarly, T cell signaling molecules ZAP-70 (Sol-Foulon et al., 2007) and ADAP (Wei et al., 2013) appeared crucial for proper VS formation. In this initial description of the VS, likeness to the IS was even extended to the characterization of supramolecular structures, Env-CD4 interactions providing a stop signal to activated target T cells and initiating the segregation in a central cluster sSMAC (central SupraMolecular Activating Clusters) of Env-CD4 surrounded by a ring-shaped pSMAC of ICAM-1 (peripheral SupraMolecular Activating Clusters) (Vasiliver-Shamis et al., 2008) stabilizing a rather static VS.

Although IS and VS share some elements and morphologic features related to cell polarization, VS appears to resemble more a modified migrating process than a stable junction. Live imaging indeed provided a new view of the VS, progressively unveiling morphologic and dynamic discrepancies between IS and VS. First, kinetic descriptions indicated that VS could establish in approximately 10-15 minutes (Chen et al., 2007; Martin et al., 2010; Rudnicka et al., 2009) and persist for at least one hour (Hubner et al., 2009; Martin et al., 2010). Although persisting, this contact was not static but rather dynamic, with infected T cells displaying a polarized and migrating phenotype (Chen et al., 2007). HIV-1 Gag was indeed shown to drive viral assembly at the uropod of T cells, enabling virus spreading through the formation of uropod-mediated cell contacts (Chen et al., 2007; Llewellyn et al., 2010). In contrast to the classical IS, the morphology of the VS then appeared as an open (Jolly et al., 2011; Martin et al., 2010), diverse (Rudnicka et al., 2009) and relatively flexible structure, alternating between circular ring shape (Rudnicka et al., 2009) and button shape (Hubner et al., 2009). VS can even sometimes establish between one donor and several recipient cells at the same time in so-called polysynapses depicted in Figure 13D (Rudnicka et al., 2009). Some variations of the VS such as nanotubes and filopodia also support HIV-1 transmission between T cells. Regarded as « stretched synapses », nanotubes are fine membrane cytonemes which connect the plasma membrane of infected and target T cells as shown in Figure 13B. As in the VS, Gag, Env and CD4 are accumulated in the contact zone between the cytonemes where viral particles are transferred to the recipient cell (Sowinski et al., 2008). In addition, target cells emit filopodial bridges able to capture HIV-1 virions through Env-CD4 interactions. Viruses then divert the actin-based retrograde flow to

surf along the filopodia toward the body of the target cell as shown in Figure 13C (Sherer et al., 2007).



**Figure 12. VS and cousins**

**A.** First reported observation of a virological synapse formed between a Jurkat<sub>LAI</sub> donor cell and a primary CD4<sup>+</sup> T target cell stained for Env in green and CXCR4 in red and observed by confocal microscopy after 30 min co-incubation (from (Jolly et al., 2004)). Arrow points an Env cluster at the surface of the target cell. **B.** HIV-1 Env (green) and Gag (red), colocalize (yellow), in nanotubes connecting Jurkat<sub>LAI</sub> T cell to uninfected Jurkat T cell in blue after 1.5–2 h co-incubation (from (Sowinski et al., 2008)). Arrow points a cluster of viral material transferred along the nanotube. **C.** Cos-1 cell generating infectious Gag-CFP HIV-1 (green), forms filopodes toward a non-infected target XC cell expressing CXCR4 and CD4-YFP (red) (from (Sherer et al., 2007)). Arrows point HIV particles transferred along filopodia from infected toward non-infected cells. **D.** Confocal imaging 1 h after conjugate formation between an infected Jurkat cell and two target Jurkat cells (blue) shows colocalization of Gag (red) with the lipid raft marker CD59 (green) at the intercellular junction (from (Rudnicka et al., 2009)). Arrow points a cluster of viral material at the surface of the target cell.

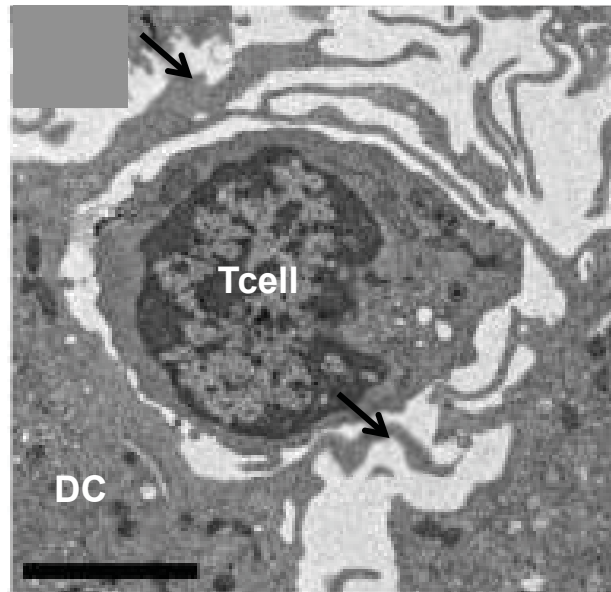
## b. The infectious synapse and the *trans*-infection process

As they patrol the peripheral tissues and migrate to secondary lymphoid organs to initiate an immune response, DCs also play a key role in the dissemination of HIV infection. DCs can then either become productively infected or only capture and convey viruses to permissive cells, both aspects relying on different cellular mechanisms (Blauvelt et al., 1997) and occurring in different time frames (Turville et al., 2004). At the initial steps of infection, DCs divert HIV-1 virions from the endolysosomal pathway to the DC-T-cell synapse (Turville et al., 2004). Called “trans-infection” (Cameron et al., 1992), this process leads to a productive T cell infection that does not require DC infection (Blauvelt et al., 1997). At this stage, mature DCs (mDCs) are more efficient than their immature counterparts in transferring HIV-1, resulting in a vigorous infection (Cavrois et al., 2007; McDonald, 2010; Wang et al., 2007). Maturation of dendritic cells, while restricting HIV replication and subsequently impeding transfer of *de novo*-produced virions, favors viral capture and infection of CD4<sup>+</sup> T cells *in-trans*. Interestingly, presence of mDCs in the vaginal mucosa could result from HIV-induced release of thymic stromal lymphopoietin (TSLP) by cervical epithelial cells, leading to increased CD4<sup>+</sup> T cell infection *in-trans* (Fontenot et al., 2009).

Secondly, a later phase of transfer arises through *de novo* production of HIV-1 by productively infected immature DCs (Turville et al., 2004).

A spectacular morphologic feature of mDC-T cell infectious synapse is the literal engulfment of the target T cell in large “sheets” formed by membranes processes emerging from the dendritic cell, as observed by cutting-edge optic and electronic imaging techniques (Felts et al., 2010) and depicted in Figure 13. HIV receptor CD4 and co-receptors CCR5 and CXCR4 are recruited to the interface while the mDCs concentrates HIV to the same region, facilitating HIV transmission by locally concentrating virus, receptor, and co-receptor (McDonald et al., 2003). Quantitative studies indicate that the vast majority of virions thus concentrated and transferred *in-trans* arise from deep invaginations of the plasma membrane (Cavrois et al., 2007; Felts et al., 2010), most likely responsible for resistance of trans-infection to trypsin treatment (Ayehunie et al., 1995) and for the preservation of HIV infectivity for prolonged periods of time (de Witte et al., 2007; Geijtenbeek et al., 2000). mDCs do not represent the only mediators of trans-infection, as many other non-permissive cell types such as spermatozoa (Ceballos et al., 2009), epithelial cells (Bomsel, 1997; Ganor

et al., 2010), B cells (Moir et al., 2000) and follicular dendritic cells (Banki et al., 2005) also prove good carriers for HIV-1 transmission to susceptible cells.



**Figure 13. Viral synapse.**

*Ion Abrasion-Scanning Electron Microscopy (IA-SEM) imaging of a contact between an HIV-1 pulsed dendritic cell and an autologous T cell cocultured for 1h. The T cell is enveloped by sheet-like membrane protrusions originating from the dendritic cell, pointed with black arrows (Scale bar: 3  $\mu$ m) (from (Felts et al., 2010).*



### c. Properties of HIV-1 transfer across synapses

Overall, either involving direct T-T junction called virological synapses or implying the participation of other permissive or non-permissive cell types in infectious synapses, transmission *via* cell contacts appears crucial for viral dissemination. Thus, cell-to-cell spread hijacks normal adhesive and interactive functions of immune cells to disseminate large amounts of viral antigen from cell-to-cell, providing HIV-1 with major features summarized in Table 3.

First, synaptic spread of HIV-1 allows a rather rapid transfer of viral material within one hour both in the context of virological (Chen et al., 2007; Martin et al., 2010) and infectious synapse (Ganor et al., 2010).

Second, local accumulation of viral material can be observed at the interface between donor and target T cells (Hubner et al., 2009; Jolly et al., 2011) as well as between donor DC and target T cell (Ganor et al., 2010). As efficiency of transfer correlates to the amount of Gag accumulated at cellular interface (Zhong et al., 2013), VS-mediated spread of HIV-1 is likely to promote simultaneous transfer of numerous viral particles. Consistent with this, multiple integration events are observed *in vivo* in CD4<sup>+</sup> splenocytes (Del Portillo et al., 2011) and VS yields 4 times more integration events than cell-free infection *in vitro* (Russell et al., 2013). Similarly, macrophage to T cell transfer of HIV-1 leads to a high multiplicity of infection (Duncan et al., 2014). These findings have implications for reported increased efficiency of HIV-1 synaptic transfer (Chen et al., 2007; Dimitrov et al., 1993; McDonald et al., 2003), for potential recombination amongst distinct viral genomes, but also for reduced sensitivity of HIV-1 to antiretroviral treatment (ArT). HIV-1 synaptic transmission indeed appears less sensitive to ArT both upon transfer from macrophages to T cells (Duncan et al., 2014) and between T cells (Agosto et al., 2014; Gupta et al., 1989; Titanji et al., 2013; Zhong et al., 2013), possibly due to an increased multiplicity of infection (Sigal et al., 2011).

Additionally, although the VS represents an open structure partially accessible to NAb, synaptic infection reduces HIV-1 sensitivity to certain NAb both in the context of T-T transmission (Chen et al., 2007; Malbec et al., 2013; Martin et al., 2010; Schiffner et al., 2013) and upon transmission from macrophages to T cells (Duncan et al., 2013).

Finally, although an excess of tetherin seems to restrict HIV-1 cell-to-cell transmission between T cells (Casartelli et al., 2010), VS-mediated HIV-1 spread mostly circumvents tetherin basal retention both through T-T transfer (Jolly et al., 2010; Zhong et al., 2013) and upon transmission from DCs to T cells (Coleman et al., 2011). Similarly, HIV-1 transmission

across the virological synapse impairs restriction induced by the expression of TRIM5 $\alpha$  from rhesus macaque (Richardson et al., 2008)

Overall, HIV-1 synaptic transmission circumvents barriers otherwise impairing cell-free infection (Zhong et al., 2013), without exhibiting a total resistance to them.

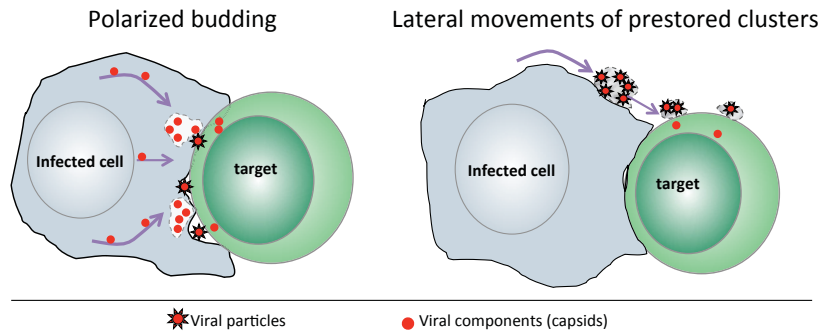
**Table 3. Major features of HIV-1 cell-associated infectivity.**

Feature	Virological synapse	Infectious synapse DC $\rightarrow$ T M $\rightarrow$ T
Viral transfer within 1 hour	(Chen et al., 2007) (Martin et al., 2010)	DC-T(Ganor et al., 2010)
Local viral accumulation	(Jolly et al., 2011) (Hubner et al., 2009)	DC-T(Ganor et al., 2010)
High multiplicity of infection	(Russell et al., 2013) (Del Portillo et al., 2011)	M-T(Duncan et al., 2014)
Reduced sensitivity to ArT	(Agosto et al., 2014) (Gupta et al., 1989) (Titanji et al., 2013) (Zhong et al., 2013)	M-T (Duncan et al., 2013)
Reduced sensitivity to NAbs	(Chen et al., 2007) (Malbec et al., 2013) (Martin et al., 2010) (Schiffner et al., 2013)	M-T (Duncan et al., 2014)
Circumvention from restriction factors	Tetherin: (Jolly et al., 2010) (Zhong et al., 2013) TRIM5 $\alpha$ : (Richardson et al., 2008)	DC-T (Coleman et al., 2011)

*DC =dendritic cell ; M = macrophage*

### 3. Nature of the viral material transferred at cellular junctions: polarized budding or prestored viral clusters?

The virological synapse is usually seen as a cellular junction enabling the polarized budding of viral particles toward the interface with the target cells (Jolly et al., 2011). For MLV for instance, directed assembly towards the site of contact is even documented (Jin et al., 2009). However, MLV transmission also involves lateral movements of surface-bound virions, retained at the cell surface through host cell surface glycosaminoglycans (GAGs) (Sherer et al., 2010). HIV-1 viral material transferred at the synapse could as well represent budded viruses bound to the cell surface that would be drawn towards the cell-cell contact zone (Jin et al., 2010).

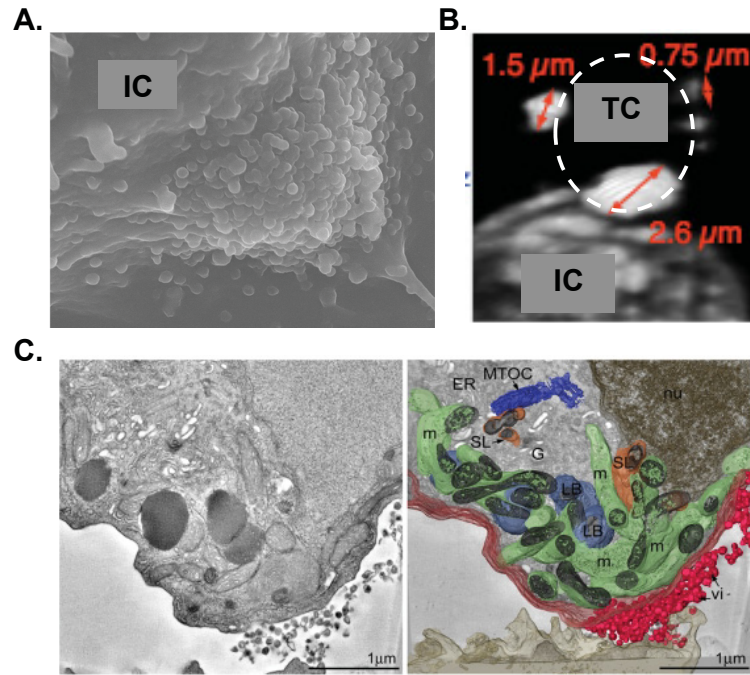


**Figure 14. Polarized budding VS lateral movements of surface-bound viral clusters.**

*Models of retroviral transmission at the virological synapse: polarized budding and assembly at the interface versus lateral movements of surface-bound viral clusters*

As depicted in Figure 15, some elements seem to indicate that HIV-1 infected T cells are able to prestore viral clusters (Rudnicka et al., 2009) and transfer them at the virological synapse (Hubner et al., 2009; Jolly et al., 2011). Although those clusters could be composed of budded viral particles retained at the surface of the infected cell, they are currently mostly seen as budding platforms that accumulate under the plasma membrane of infected cells (Ivanchenko et al., 2009; Jouvenet et al., 2008) undergoing a contact-induced polarized budding upon interception with a target cell (for review see (Feldmann and Schwartz, 2010)).

Regarding the maturation state of virions transferred at the VS, some claim that the VS promotes the transfer of immature viral particles to acceptor cells, where they undergo protease-dependent maturation within endosomal compartments (Dale et al., 2011). On the contrary, HIV-1 cell-free infection is relying on viral fusion at the plasma membrane of target cells (Stein et al., 1987). It is thus possible that the nature of viral particles transferred across the VS differs from the one transferred in a cell-free manner, leading to different internalization routes in target cells, as well as different maturation processes. As maturation triggers subsequent Env conformational changes (Wyma et al., 2004), transfer of immature viral particles across the VS could contribute to their reduced sensitivity to NABs.



**Figure 15. Local accumulation of viral particles at the surface of infected cells.**

*A. Scanning electron micrograph showing aggregates of budded viral particles at the surface of HIV-1 infected primary CD4<sup>+</sup> T cell (IC) (from (Rudnicka et al., 2009)). B. Snapshot of a 3D laser scanning confocal sequence showing HIV Gag-iGFP-expressing Jurkat cell (IC) synapsed with a CD4<sup>+</sup> target cell (dotted white line, TC). Large Gag puncta with a diameter ranging between 0.75 and 2.6  $\mu\text{m}$  are transferred to the target cell and remain stable at its surface for at least 13h (from (Hubner et al., 2009)). C. Tomogram (left panel) and 3D surface rendering (right panel) of a synapse formed between an infected Jurkat T cells and a primary CD4<sup>+</sup> T cells display aggregates of budded viral particles transferred to the target cell (ER = endoplasmic reticulum, SL = secretory lysosomes, G = Golgi, m = mitochondria, LB = lipid bodies, MTOC = Microtubules organizing center) (from (Jolly et al., 2011)).*

## V. Microbial biofilms: a vehicle for collective dissemination

Initially described for bacteria in the early 1980s, biofilms have been more recently shown to sustain retroviral spread at intercellular junctions as well.

### 1. General features of bacterial biofilms

Described by pioneer microscopic studies in the early 1980s (McCoy et al., 1981), bacterial biofilms are composed of aggregates of bacteria cocooned in a self-produced extracellular matrix (ECM), which accounts for ~90 % of their biomass. This ECM usually contains extracellular polysaccharides (EPS), carbohydrate-binding proteins, amyloid fibrils, pili, flagella and extracellular DNA (eDNA) (Kostakioti et al., 2013). An example of the morphology of bacterial biofilms is given in Figure 16.

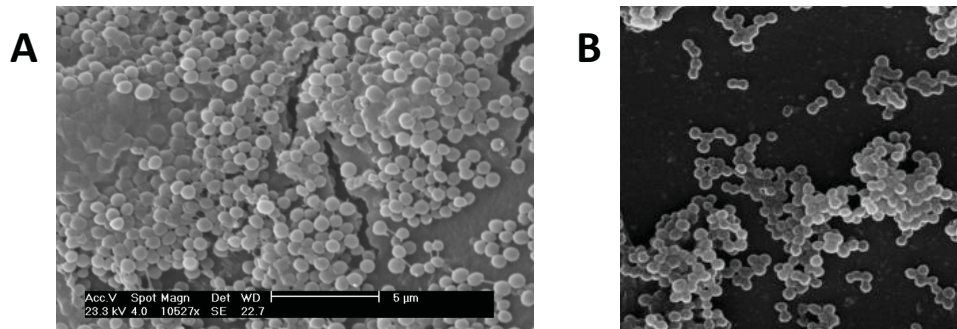
The first step in bacterial aggregation and subsequent biofilm maturation involves the introduction of bacteria to a surface (Kostakioti et al., 2013). Upon intercepting the surface, adherence is mediated by extracellular adhesive appendages and secreted adhesins. In addition, host ECM proteins such as laminin, plasminogen, fibronectin (Olsen et al., 1989), collagen (Mohamed et al., 2006) and galectin-3 (Fowler et al., 2006) can serve as a scaffold to initiate biofilm formation. Several enterococcal adhesins mediate adherence to eukaryotic ECM components. Examples include SagA, Acm (*E. faecium*), Curli (*E. coli*) and Ace (*E. faecalis*), which binds collagen (Mohamed et al., 2006).

Ensues the establishment of a “group behavior” that involves a communication strategy called “quorum sensing” (for review see (Holm and Vikstrom, 2014)). Although the formation of a biofilm appears cost-effective, multicellularity equips bacteria with an arsenal of properties that enable their survival in unfavorable conditions (Kostakioti et al., 2013).

Within bacterial biofilms, the ECM indeed not only holds the constituent cells in close proximity but also creates a bidirectional barrier to the environment. This barrier retains water, enzymes and nutrients close to the cell surface. In addition, this barrier protects the biomass from desiccation, predation, oxidizing molecules, radiation, and other damaging agents (Kostakioti et al., 2013). In the environment, EPS has indeed been reported to provide protection from a variety of environmental stresses, such as UV radiation, pH shifts, osmotic shock, and desiccation (Davey and O'Toole G, 2000).

Inside the host, the ECM protects bacteria from exposure to innate immune defenses such as opsonization and phagocytosis (for review see (Foster, 2005)) and limits the

penetration of antibiotic treatments (for review see (Stewart, 2002)). In addition, as the vast majority of biofilms in environmental habitats contain multiple bacterial species and in many cases also fungi, algae, and protozoa (Thallinger et al., 2013), interspecies interactions can promote the spread of drug-resistance markers and other virulence factors



**Figure 16. Morphology of bacterial biofilms.**

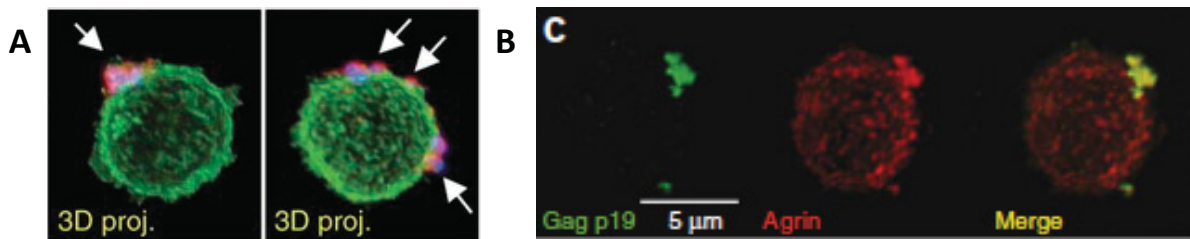
*A-B. Scanning Electron Micrographs of Staphylococcus aureus biofilm show clusters of bacteria blanketed with a mesh of extracellular matrix (from (Qin et al., 2014)).*

## 2. Viral biofilms

Human lymphotropic retrovirus Human T-cell Leukemia Virus Type I (HTLV-1) is reported to propagate between T cells via a polarized budding occurring at the virological synapse (Igakura et al., 2003). However, the paradigm of HTLV-1 polarized budding at the virological synapse has been recently revisited. Our laboratory has indeed shown that Human T Cell Leukemia Virus Type 1 (HTLV-1) infectious viral particles are mainly transmitted at the virological synapse through the transfer of a “viral biofilm” (Figure 17) (Pais-Correia et al., 2010). HTLV-1 prestored extracellular viral assemblies have been called “viral biofilms” because their structural and functional features were strongly reminiscent of those of bacterial biofilms (Thoulouze and Alcover, 2011) described in the previous paragraph. Pais-Correia *et al.*'s work on HTLV-1 cell-to-cell transmission defined viral biofilms as follows.

**Viral biofilms: extracellular colonies of infectious viral particles encased in a cocoon enriched in extracellular matrix and linker proteins produced by the infected cell. Viral biofilms locally increase viral infectious titers, play a crucial role in viral transmission and potentially protect the viral particles they contain.**

HTLV-1 biofilm ECM components include the heparan sulfate agrin, galectin 3 as well as collagen, the expression of which is up-regulated by viral infection. HTLV-1 biofilm represents a highly adhesive infectious structure that potentially protects and clusters HTLV-1 virions, pre-storing them at the surface of the infected T cell. HTLV-1 enables a quick transmission of HTLV-1 virions to the target T cells and is responsible to as much as 80 % of HTLV-1 cell-to-cell transmission.



**Figure 17. Morphology of HTLV-1 biofilm.**

*A. Confocal imaging of HTLV-1 infected T cells (ConA, green) carrying a viral biofilm (HTLV-1 gag, blue ; serum targeting HTLV-1 env, red) (from (Pais-Correia et al., 2010)). B. Confocal imaging of HTLV-1 infected T cell carrying a viral biofilm staining positive for HTLV-1 Gag p19 (green) and agrin (red) (from (Pais-Correia et al., 2010)).*

Description of HTLV-1 biofilm thus challenges the model of a polarized budding in the synaptic cleft upon formation of a virological synapse. As viral particles are already exposed at the surface of the infected cell, one can wonder whether the presence of the producing cell is dispensable for biofilm infectivity. Importantly, this discovery also raised the question whether ability to form a viral biofilm is shared by other human retroviruses, especially HIV-1, which represents an important public health threat?

Of note, HTLV-1 disseminates exclusively through the formation of cell-cell junctions. On the contrary, HIV-1 transmission is “bimodal”, as it involves both the diffusion of cell-free viral particles and the formation of intercellular junctions. Thus, it remains to be determined whether viruses displaying a “bimodal transmission” possess the ability to form a viral biofilm. Interestingly, HIV-1 infection displays an intricate interplay with the ECM. The following chapter will develop features of this interplay that could be relevant for the formation of a viral biofilm to shelter HIV-1 cell-associated infectivity.

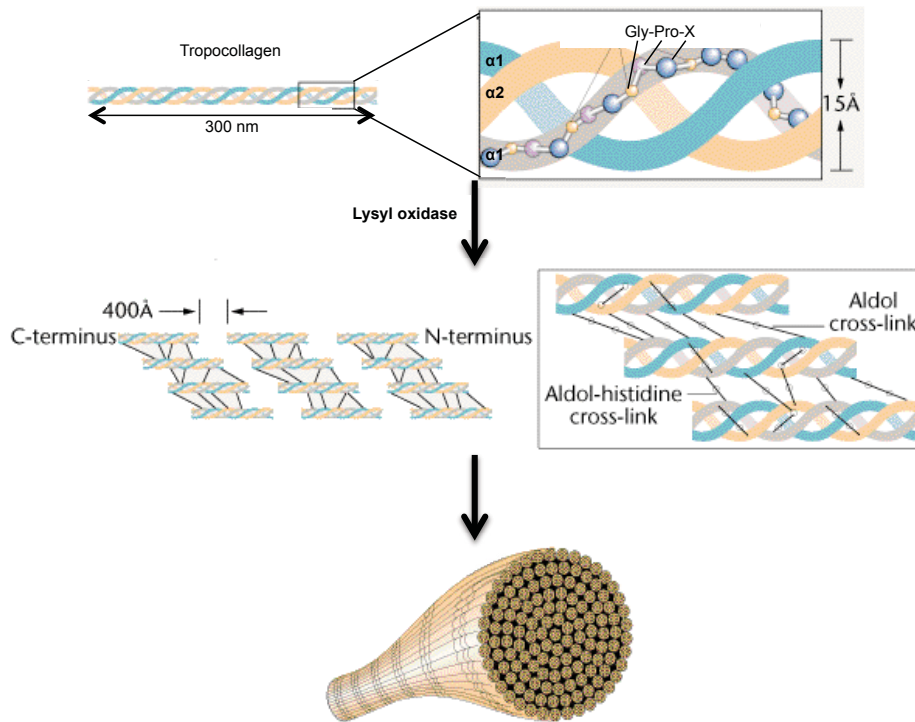
## **VI. Extracellular matrix in HIV-1 infection**

### **1. Overview on ECM main components**

Highly organized, glycosylated and insoluble, the ECM exists in three main forms: the basement membranes, the interstitial matrix and the pericellular matrix. With a few exceptions, all cells are surrounded by a pericellular matrix that gives mechanical support by binding cells together but also represents a biochemical barrier around the cell. This docking facility provides an interface for imports and exports with the cell, where chemical signaling can take place. The ECM is mainly composed of highly conserved, often oligomerized, glycosylated proteins. ECM major components include collagens and heparan sulfate proteoglycans. In addition, adhesion proteins are crucial ECM-associated proteins involved in cell-ECM interactions.

Collagens exist in at least 16 types, yet the most represented types are fibrillar collagens I, II and III, as well as reticulated collagen IV. As depicted in Figure 18, collagen basic structural unit is a triple-stranded helical tropocollagen molecule composed of two  $\alpha 1$  and one  $\alpha 2$  coiled-coiled chains. In each chain contains, amino acids make up the characteristic repeating motif Gly-Pro-X, where X can be any amino acid. In fibrillar collagen, tropocollagen molecules pack together side by side and one following the other, with a small gap separating the “head” of one collagen molecule from the “tail” of the next. The unique properties of each type of collagen are mainly due to segments that interrupt the triple helix. Covalent bonds catalyzed by lysyl-oxidase stabilize side-by-side interactions between adjacent tropocollagen molecules (Lodish H, 2000).



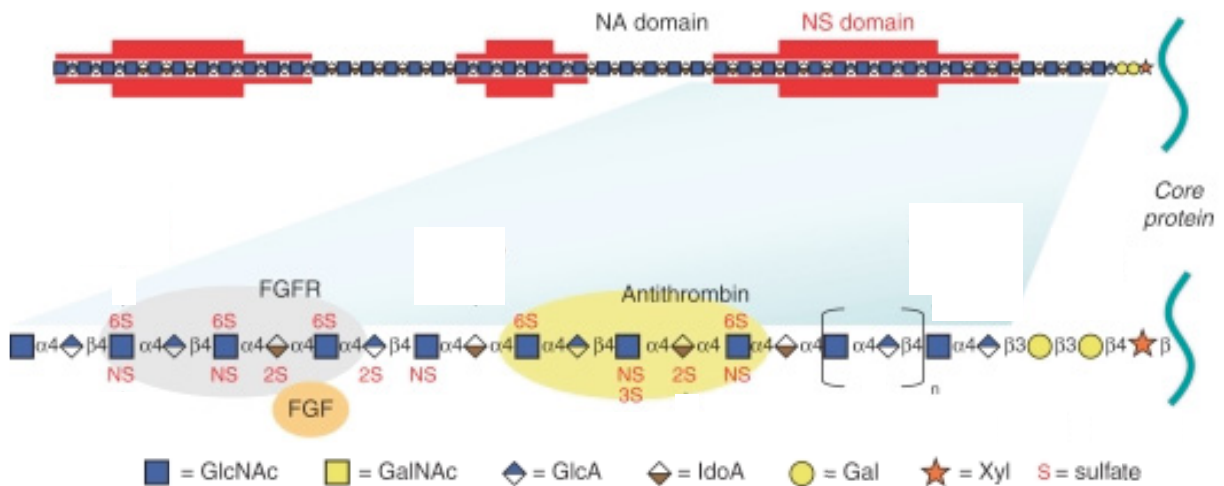


**Figure 18. Structure and organization of collagen fibrils.**

*Tropocollagen molecules are composed of 1  $\alpha 2$  chains and 2  $\alpha 1$  chains, each containing 1,050 amino acids displaying Gly-Pro-X repeats. Lysyl-oxidase catalyzes the cross-linking of tropocollagen side-chains to form collagen fibrils. From (Lodish H, 2000).*

Proteoglycans are glycoproteins carrying long Glycosaminoglycans (GAGs), unbranched polysaccharides consisting of repeated disaccharide units. GAG chains encompass Chondroitin, Dermatan, Keratan and Heparan sulfates. Cells elaborate a relatively small set of about 17 Heparan Sulfate Proteoglycans (HSPGs) that fall into three groups according to their location: membrane HSPGs, such as syndecans and glycosylphosphatidylinositol-anchored proteoglycans (glypicans), secreted HSPGs (agrin, perlecan, type XVIII collagen) and the secretory vesicle proteoglycan serglycin. As depicted in Figure 19, HSPGs contain one or more covalently attached heparan sulfate (HS) chains, composed of an alternation of 40 to 300 glucuronic acid GlcA and N-acetyl-glucosamine GlcNAc. Unmodified N-acetylated (NA) domains interspace clusters of N-sulfated (NS) domains in which sulfates are substituted to the acetyl groups of GlcNAc residues. NS domains make up binding sites for protein ligands such as antithrombin, FGF and FGF receptor, but also for chemokines, like SDF-1. Binding capacities of membrane HSPGs such as syndecan-2, -4 and glypicans mediate the endocytic uptake of some ligands (Christianson and Belting, 2014). In addition, HSPGs bind other ECM proteins, and among them

fibronectin, laminin, vitronectin, thrombospondin, and some fibrillar collagens. Finally, HSPG cooperate or interact with cell adhesion molecules such as integrins and L-selectin to mediate either cell-ECM or cell-to-cell interactions (Sarrazin et al., 2011).



**Figure 19. Typical structure of an heparan sulfate chain.**

*Bound to the core protein through a xylose residue followed by a 5-residue linker, heparan sulfates are composed of unmodified N-acetylated (NA) and N-sulfated (NS) domains. Each of these domains consists in an alternation of glucuronic acid and N-acetyl-d-glucosamine. Within NS domains, the acetyl group of N-acetyl-d-glucosamines is substituted by a sulfate group. NS domains represent binding sites for ligands such as FGFR, FGF or antithrombin among others (from (Sarrazin et al., 2011)).*

Finally, cell adhesion molecules represent crucial ECM-associated proteins, as they mediate cell binding and interaction with the ECM. Among them, integrins are the major ECM binding proteins, as they recognize short peptide sequences (e.g., RGD) present in many ECM proteins (Cox et al., 2006). Selectins and adhesion molecules from the immunoglobulin superfamily (N-CAM, ICAM, VCAM,...) also mediate cell interaction with its surrounding matrix (e.g. (Languino et al., 1995)). Similarly, transmembrane heparan sulfate CD44 enables cell adhesion to fibronectin (Jalkanen and Jalkanen, 1992), hyaluronic acid (Lesley and Hyman, 1992) as well as laminin and collagen IV (Ishii et al., 1993)

In HIV-1 pathophysiology, extracellular matrix (ECM) components, and especially collagen, are primarily characterized for their role in the establishment of fibrotic damage in lymph nodes (Estes, 2009). ECM role in HIV-1 transmission to target cells, its modulation in HIV-1 infected T cells and its ability to interact with HIV-1 virions represent more cryptic aspects of its implication in HIV-1 physiopathology.

## 2. ECM participation in the transmission of HIV-1 viral particles

Current knowledge on ECM contribution to HIV-1 transmission mainly focuses on its role as an attachment factor, favoring both virus carriage by non-permissive cells and virus binding to target cells, an aspect reported for several other viruses (Kunz, 2009; Richards et al., 2014).

### a. ECM components participate in HIV-1 carriage by non-permissive cells

ECM components contribute to many aspects of HIV-1 carriage, from initial transmission stages from one individual to another to dissemination throughout the body.

ECM components participate in HIV-1 initial entry steps at mucosal barriers. HIV-1-infected cell-induced transcytosis indeed depends on integrins but also on the HSPG agrin, that displays the ability to bind HIV-1 gp41 and acts as an HIV-1 attachment receptor at the apical surface of epithelial cells (Alfsen et al., 2005).

Following entry at mucosal barriers, HIV-1 is efficiently captured by DCs in the periphery and transported to secondary lymphoid organs rich in HIV-1 target T cells. Although DC-SIGN binds HIV-1 gp120/gp41 (Spear et al., 2003) and participates in the infectious synapse between DCs and CD4<sup>+</sup> T cells (Arrighi et al., 2004), promoting efficient T cells *trans*-infection (Geijtenbeek et al., 2000), viral capture by DCs is mainly mediated by heparan sulfates. Syndecans indeed interact with HIV-1 gp120 and thus enable efficient infection of permissive cells in *trans* (Bobardt et al., 2003). More precisely, siRNA silencing of syndecan-3 inhibits HIV-1 transmission by immature DCs, suggesting syndecan-3 as a new target for the development of microbicides limiting HIV-1 *trans*-infection (de Witte et al., 2007). ECM components participate in the carriage of HIV-1 at the surface of other non-permissive cells. Heparan sulfates syndecans 3 and 4 hence represent HIV-1 attachment receptors to spermatozoa (Ceballos et al., 2009) complementary to CCR5 (Muciaccia et al., 2005) and mannose receptors (MRs) (Bandivdekar et al., 2003; Ceballos et al., 2009).

ECM components further potentiate HIV-1 transmission by carrier cells through a direct increase in the half-life of infectivity of HIV-1 viral particles. HIV-1 attachment to syndecan 2 at the surface of non-permissive cells hence preserves HIV-1 infectivity for over 5 days (Bobardt et al., 2003). Of note, *in vitro* addition of multimeric fibronectin has similar effects

(Greco et al., 2002), indicating this property as a shared and intrinsic feature for ECM components.

Overall, through increased attachment to carrier non-permissive cells and preservation of the half-life of infectivity, ECM components favor HIV-1 dissemination by non-permissive cells.

### b. ECM components favor HIV-1 attachment and fusion to target cells

As already described, the low efficiency of virion binding to target cells contributes to HIV-1 poor infectivity (Thomas et al., 2007). However, this process is facilitated by several factors and among them ECM components.

HIV-1 binding to primary CD4<sup>+</sup> T cells is indeed highly dependent on sulfated HS expressed at the surface of target cells (Ohshiro et al., 1996). More precisely, HS participate in the late stages of the virus-cell attachment process following gp120 conformational changes induced by CD4 binding (Vives et al., 2005).

HIV-1 attachment to target cells is also enhanced by soluble ECM components. Strong potentiator of lentiviral vector binding (O'Neill et al., 2010), soluble fibronectin binds HIV-1 viral particles and facilitates its attachment to target cells, either in synergy with amyloids from semen (Roan et al., 2014) or in cooperation with cell-surface proteoglycans in the case of multimeric fibronectin (Tellier et al., 2000). Similarly, HIV-1 binding to target cells is potentiated by some galectins, which represent soluble ECM-affiliated lectins. Galectin-1 hence binds clusters of complex-type glycans on HIV-1 gp120 and cross-links gp120 to host CD4, accelerating HIV-1 entry process into susceptible cells by 40-fold (Sato et al., 2012). Also enhancing HIV-1 entry into target cells, galectin-9 does not bind HIV-1 virions but rather stabilizes Protein Disulfide Isomerase (PDI) activity at the cell surface. As PDI catalyzes HIV-1 Env rearrangements required for fusion, ECM-affiliated galectin-9 favors HIV-1 entry into target cells (Bi et al., 2011).

In all examples described above, ECM proteins facilitate HIV-1 CD4-dependent entry. In some specific examples however, ECM components even affect HIV-1 initial entry steps, providing HIV-1 with alternative receptors to CD4. For instance, galactosyl ceramides appear to enable HIV-1 entry into epithelial cells (Delezay et al., 1997).

ECM components hence favor HIV-1 attachment to target cells, enabling subsequent fusion and infection.

## 2. ECM components from HIV-1 infected T cells

Little is known about T cell ECM and its physiological roles. However, T cell activation deeply modulates ECM expression patterns (see Appendix 2) and some ECM proteins such as agrin are reported to participate in immune synapses formation (Khan et al., 2001). Both through direct modulation by accessory proteins and through the induction of a peculiar activation state, HIV-1 infection also reshuffles T cell ECM expression profiles and HIV-1 particles produced in T cells appear to associate with some ECM components.

Direct modulation of T cell ECM patterns by HIV-1 mostly implicates viral transactivator Tat. As it recruits several host regulators of gene expression, Tat indeed directly amplifies the expression of laminin, fibronectin and collagen I and III (McArthur et al., 2001), possibly through TGF $\beta$  activation (Taylor et al., 1992). In addition, Tat effect is not limited to productively infected cells, as secreted Tat is able to enter target cells. Interestingly, Tat uptake is mediated by cell surface heparan sulfates proteoglycans (HSPG) (Tyagi et al., 2001) that bind Tat basic region (Chang et al., 1997). Following uptake, Tat is likely to trigger similar ECM modulations in non-infected bystander cells.

In addition, HIV-1 infection alters T-cell activation profile that is physiologically linked to a reshuffling of ECM genes expression (see Appendix 2). Engagement of CD4, CD28 and TCR at T cell surface by virion-associated gp120, CD86 and MHC resp. induces activation of Src kinases and of the early T-cell receptor (TCR) signaling machinery (Vasiliver-Shamis et al., 2009), leading to T cell activation and/or differentiation (Esser et al., 2001). In addition, Nef modulates tyrosine-phosphorylation patterns (Thoulouze et al., 2006), and triggers Lck and Erk1/2 activation (Wolf et al., 2008).

Overall, either directly or through T cell activation, HIV-1 infection induces a deep modulation of about 120 ECM genes in infected T cells, as shown in Appendix 2. Among them, collagen, already reported to be overexpressed in HIV-1–infected lymphatic tissues (Smith et al., 2010), is consistently found overexpressed in different forms.

Upon viral particle assembly and budding, HIV-1 interacts and associates with a broad range of host proteins and ECM proteins are no exception with 65 ECM components associated to HIV-1 virions across different viral strains and cell lines used for production (see Appendix 2). Relevant to this thesis, collagen subtypes (Bregnard et al., 2013) and CD44 (Bregnard et al., 2013; Chertova et al., 2006; Stephenson et al., 2010) associate to HIV-1 virions.

T cell ECM patterns are hence deeply modulated upon HIV-1 infection and some ECM components are associating to HIV-1 virions. However, the contribution of T cell ECM to HIV-1 transmission remains to be explored.

## Position of the problem

Cell-associated infectivity represents HIV-1 more potent mode of transmission *in vitro* and might constitute HIV-1 preferred mode of dissemination *in vivo*. As CD4<sup>+</sup> T lymphocytes represent the major niche for HIV-1 replication, we focused this work on HIV-1 dissemination between T cells, involving either direct T-T interactions or the *trans*-infection process.

While DC-mediated *trans*-infection of target T cells occurs through an infectious synapse, HIV-1 transmission from infected to target T cells involves the formation of organized T-T junctions called virological synapses (VS). HIV-1 synaptic transmission is highly efficient and partially resistant to antiretroviral treatment (ArT) and NAbs. Importantly, the efficiency of HIV-1 VS-mediated transmission mostly relies on the high multiplicity of infection involved at the site of contact, yielding a high number of entries and subsequent integration events. Although cell-associated infectivity arises from the cell surface of DCs conveying HIV-1 *in trans*, the nature and location of HIV-1 material prior to its transfer at the VS remains poorly documented.

At the beginning of my thesis, addressing of HIV-1 material to the VS was described to occur *via* a contact-induced polarized budding toward the interface with the target cell. Over the ensuing years, the VS was described as a more dynamic structure involving the lateral movement of pre-formed Gag platforms associated to the uropod of T cells toward the contact zone. Those platforms could represent clusters of fully budded viral material associated to the surface of T cells. By browsing the literature, we indeed observed that the interface between infected and target T cells is colonized by clusters of mature virions.

We hypothesized that those viral clusters accumulating at the VS are crucial for HIV-1 T-cell associated infectivity. As HTLV-1 viral clusters are retained and exposed at T cell surface and subsequently transferred to target T cells in a viral biofilm enriched in ECM components, we wondered whether HIV-1 retention at T cell surface could be mediated by ECM components as well. Deeply modulated upon HIV-1 infection, the ECM indeed strongly interplays with HIV-1 virions and thus represents a potential scaffold for such HIV-1 clusters, possibly mediating their retention at T cell surface and transmission at intercellular junctions.

In this context, we addressed the following questions:

- *What is the contribution of surface-bound virions to HIV-1 cell-associated infectivity?*
- *Are HIV-1 membrane-bound virions clustered in an ECM-rich viral biofilm exposed at the surface of infected T cells?*
- *If a viral biofilm mediates HIV-1 retention at T cell surface, what is its contribution to HIV-1 transfer between T cells and in the trans-infection process?*
- *What properties does this viral biofilm confer to HIV-1 cell-to-cell transmission? More precisely, does HIV-1 biofilm contribute to already characterized properties of HIV-1 transmission by cell contacts, such as increased infectivity and decreased sensitivity to ArT and NAbs?*

Using a combination of fluorescence and electronic microscopy techniques, we explored the presence of HIV-1 viral biofilm for chronically infected T cell lines as well as for primary CD4<sup>+</sup> T cells infected *in vitro* with HIV-1 laboratory strains and primary isolates. Importantly, we confirmed the relevance of our findings by the study of CD4<sup>+</sup> T cells from HIV-1 patients. Functional assays enabled us to delineate the contribution of HIV-1 biofilm both for direct T-T transmission and for indirect transmission involving DC-mediated infection in *trans*. Further, we analyzed the role played by biofilm-ECM components in the efficiency of HIV-1 transmission. Finally, we addressed the relevance of biofilm-mediated transmission in the reported increased infectivity and decreased sensitivity to ArT and NAbs of HIV-1 transmission by cell contacts.

This work will be presented in the following Results section. The corresponding scientific manuscript will soon be submitted.





## **RESULTS**



# I. HIV-1 T-cell associated infectivity mostly resides in a viral biofilm

## 1. T-cell associated infectivity is carried at the cell surface

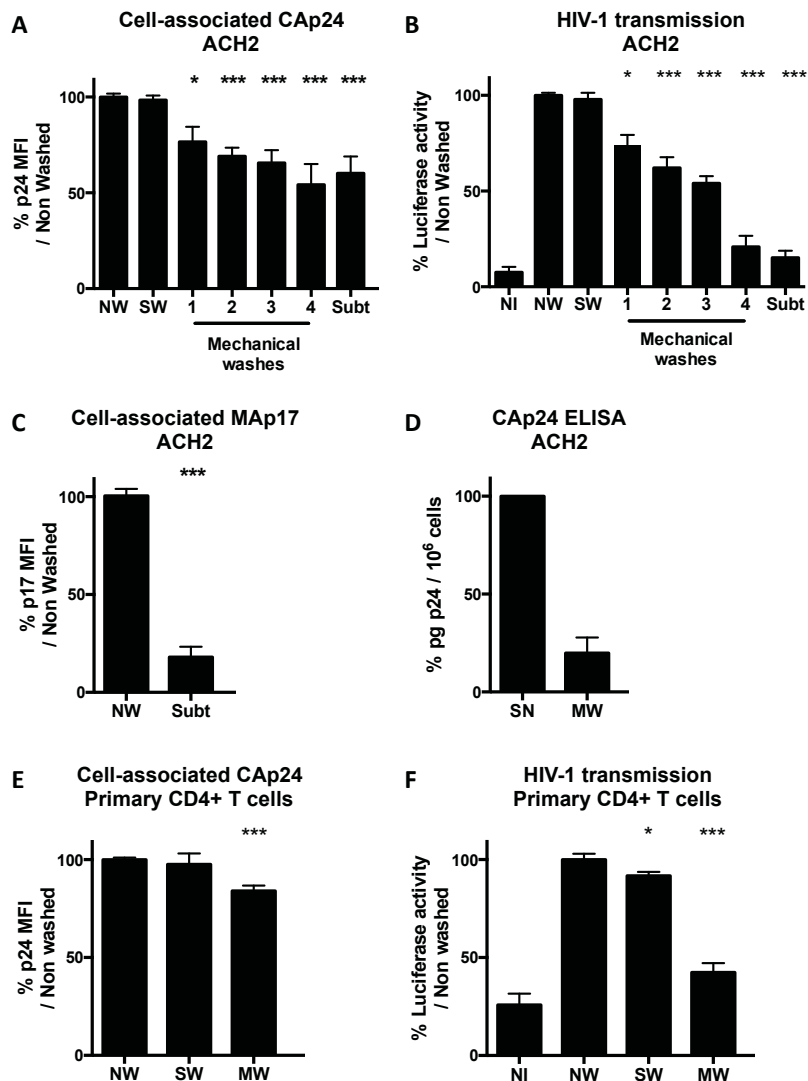
As most HIV-1 infectious material transferred during *trans*-infection originates from membrane invaginations of DCs (Cavrois et al., 2007), we wanted to assess the quantitative contribution of cell-surface viral material in HIV-1 intercellular transmission between CD4+ T lymphocytes.

We subjected HIV-1 infected T lymphocytes to gradual mechanical washes and subsequently measured remaining cell-associated infectivity. Mechanical washes of HIV-1 chronically infected ACH2 cells led to up to 50% reduction in cell-associated CAp24 (Figure 20C), resulting in up to 80% decrease in cell-associated infectivity (Figure 20D). Interestingly, progressive decrease in cell-associated CAp24 correlated to gradual decrease in cell-associated infectivity over mechanical washes 1 to 4 (Figure 20C&D). As single wash aiming at releasing viral particles loosely adhering to the surface of the cells only poorly decreased cell-associated CAp24 (Figure 20C) and resulted in unaffected infectivity (Figure 20D), most surface-associated infectivity appeared to reside in firmly adhering extracellular viral material.

As flow cytometry quantification of CAp24 does not discriminate between intracellular and extracellular viral material, we treated ACH2 cells with Subtilisin, a protease commonly used to remove surface-bound viral material. Subtilisin treatment triggered a 75% decrease in cell-associated mature viral matrix MAp17 (Figure 20E), simultaneously leading to decreases both in cell-associated CAp24 (50%) and in infectivity (80%) comparable to that yielded by mechanical removal (Figure 20C&D). Thus, 50% decrease in cell-associated CAp24 following mechanical washes of ACH2 cells corresponds to an efficient removal of surface-associated viral material.

ELISA quantifications revealed that  $213 \pm 27$  ng/ $10^6$  cells of CAp24 were released by mechanical washes, corresponding to 25% the amount of CAp24 detected in cell culture supernatant (Figure 20F). Even for wild-type viruses expressing Vpu, an unexpectedly high amount of viral material is thus retained at the cell surface. Further, Although representing only a fifth of viral material produced infected T cells, HIV-1 surface-associated material thus contributes to up to 80% of the efficiency of HIV-1 cell-to-cell transmission.

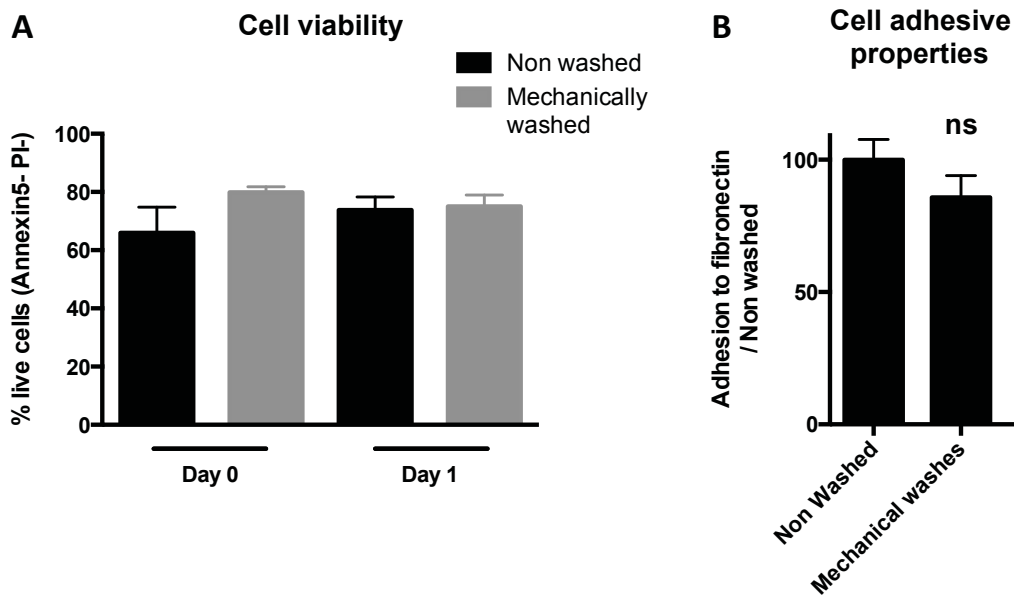
Contribution of surface-associated viral material to HIV-1 intercellular transmission was addressed for primary CD4<sup>+</sup> T cells infected with HIV-1 NL4-3. With the concern to preserve the viability of infected primary CD4<sup>+</sup> T cells, mild mechanical washes were performed, leading to a 20% decrease in cell-associated CAp24 (Figure 20A). This removal led to a 60% decrease in cell-associated infectivity (Figure 20B), confirming the crucial contribution of surface-bound viral material to HIV-1 T-T cell-to-cell transmission.



**Figure 20. HIV-1 T-cell associated infectivity mostly resides at the cell surface.**

ACH2 cells (A-D) and primary CD4<sup>+</sup> T cells infected with NL4-3 (E-F) were left non-washed (NW), washed once (SW) or subjected to gradual mechanical washes (MW) or subtilisin (Subt) treatment. A, E. Flow cytometry quantification of cell-associated CAp24, Mean Fluorescence Intensity (MFI) + SEM of triplicates from at least three independent experiments. B, F. Luciferase assay determining cell-associated infectivity using a Jurkat LTR-luciferase clone, mean + SEM of triplicates from at least three independent experiments. C. Flow cytometry quantification of cell-associated MAp17 following subtilisin treatment, MFI + SEM of triplicates from four independent experiments. D. CAp24 ELISA determining the relative amount of CAp24 per 10<sup>6</sup> cells in culture supernatant (SN) and released by mechanical washes (MW), mean + SEM from 20 independent experiments. P-values are calculated using Mann-Whitney tests (\*:  $p < 0.1$ , \*\*\*:  $p < 0.001$ ).

We considered the possibility that mechanical washes could affect features conditioning efficient cell-associated infectivity, such as T cell viability or their capacity to form intercellular junctions. However, neither cell viability (Figure 21A) nor cell adhesion capacities (Figure 21B) of infected cells were affected by mechanical treatment. Thus, reduction in cell-associated infectivity was mainly attributable to the removal of HIV-1 extracellular material from the surface of infected T cells.



**Figure 21. Mechanical washes affect neither cell viability nor adhesion capacities.**

*ACH2 cells were left non-washed or subjected to gradual mechanical washes. A. Flow cytometry quantification of cellular viability by Annexin V/PI staining at Day 0 and Day 1 after treatment, mean + SEM of triplicates. B. Assay determining adhesion to fibronectin, mean + SEM of triplicates from three independent experiments.*

## 2. HIV-1 infected T cells produce extracellular aggregates of viral material

As HIV-1 cell-associated infectivity mostly resides at the cell surface, we wanted to localize HIV-1 mature viral material in infected CD4<sup>+</sup> T lymphocytes with regard to the plasma membrane.

Confocal microscopy revealed that chronically infected J1.1 and ACH2 T cells broadly used for functional studies carry aggregates of mature HIV-1 MAp17<sup>+</sup> viral material at the surface of their plasma membrane, delineated using an anti-CD98 immunostaining that is excluded from the viral particles in non-permeabilizing conditions (Figure 22).

We then studied a model of acute infection in which primary CD4<sup>+</sup> T cells were infected *in vitro* with HIV-1 laboratory strains NL4-3 and BaL as well as primary virus isolates DH12, Bx08 and W132, reaching an infection efficiency of  $18 \pm 4$  to  $46 \pm 18\%$  of CAp24<sup>+</sup> cells depending on the virus considered (Table 4). In order to specifically stain the plasma membrane of primary CD4<sup>+</sup> T cells, we took advantage of the active exclusion of CD45 transmembrane protein from HIV-1 virions (Esser et al., 2001) and performed an anti-CD45 labeling of the plasma membrane in non-permeabilizing conditions. Confocal microscopy revealed that in every system studied, between 69 and 74% of infected CAp24<sup>+</sup> lymphocytes carry aggregates of mature HIV-1 MAp17<sup>+</sup> viral material at their surface (Table 4), located outside of CD45 membrane staining (Figure 22). Importantly, primary CD4<sup>+</sup> T cells infected both with X4-tropic strain NL4-3 and R5-tropic strain BaL carried extracellular aggregates of viral material (Table 4), thus highlighting the independence on co-receptor tropism for this ability of HIV-1 material to form viral clusters at the surface of infected CD4<sup>+</sup> T cells.

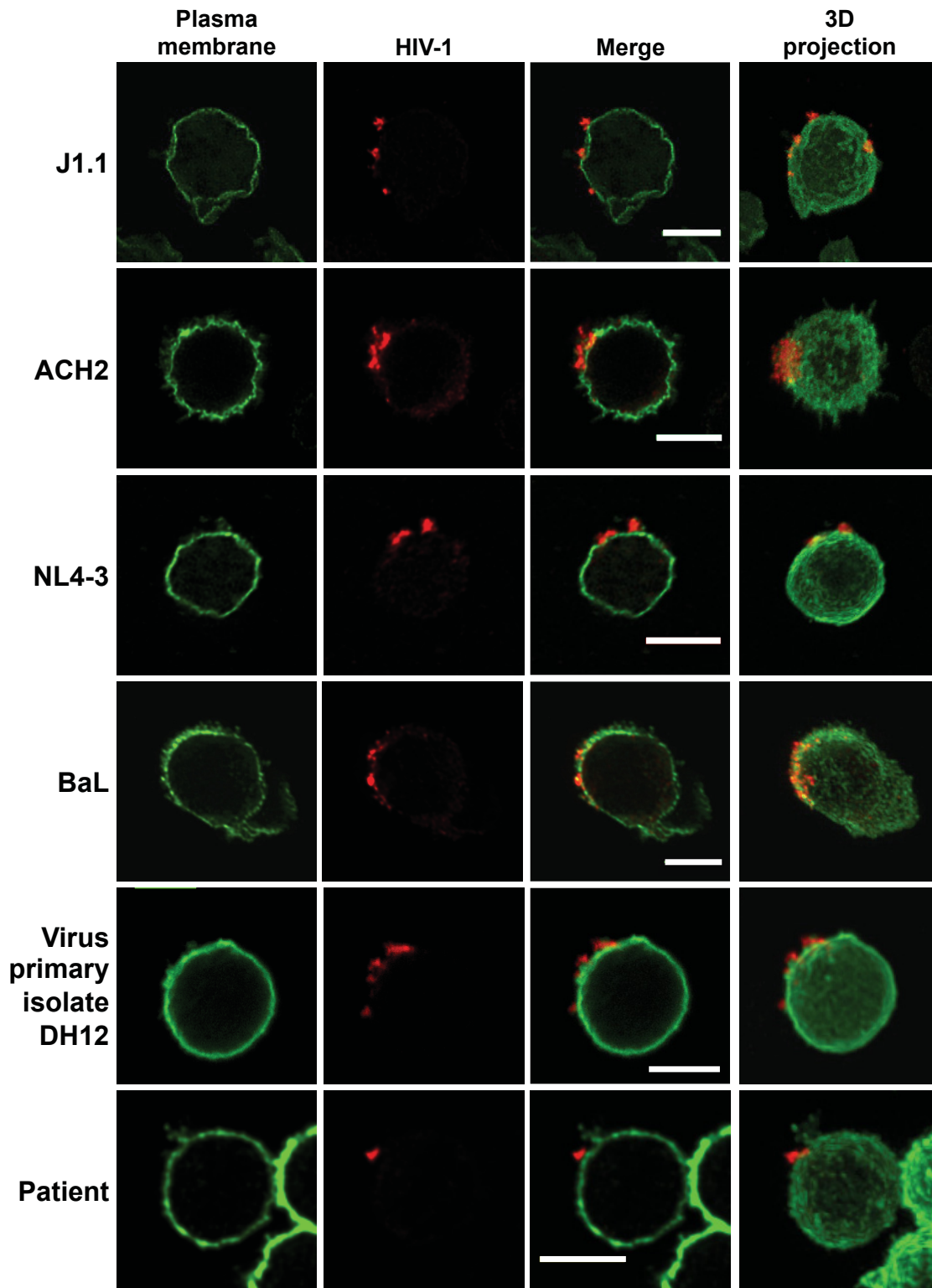
To address whether CD4<sup>+</sup> T cells from HIV-1 patients present such structures at their surface, we recruited viremic patients (viral load > 25,000, CD4 counts > 200) at the onset of HAART therapy and negatively sorted CD4<sup>+</sup> T cells from their PBMCs. Clinical characteristics of study subjects are presented in Appendix 3. Confocal microscopy revealed that, after 3 days in culture, mature MAp17<sup>+</sup> viral material was accumulating at the surface of 59% of infected CAp24<sup>+</sup> CD4<sup>+</sup> T cells from HIV-1 patients as well (Figure 22&Table 4).

**Table 4. Fraction of infected CAp24+ cells and of CAp24+ cells carrying extracellular viral material.**

<b>Cell type</b>	<b>Virus</b>	<b>Coreceptor usage</b>	<b>% p24+ cells</b>	<b>% p24+ cells carrying extracellular viral material</b>
<b>Laboratory strains</b>				
<b>ACH2</b>	NL4-3	CXCR4	89 ± 8	64 ± 13
<b>Primary CD4+ T cells</b>	NL4-3	CXCR4	26 ± 9	71 ± 3
	BaL	CCR5	46 ± 18	72 ± 2
<b>Virus primary isolates</b>				
<b>Primary CD4+ T cells</b>	Bx08	CCR5	26 ± 13	71 ± 3
	DH12	Dual tropic	35 ± 13	74 ± 7
	W132	Dual tropic	18 ± 4	69 ± 9
<b>CD4+ T cells from HIV-1 infected patients</b>		Unknown	5 ± 3	49 ± 8

*Mean ± SD of triplicates from three independent experiments.*



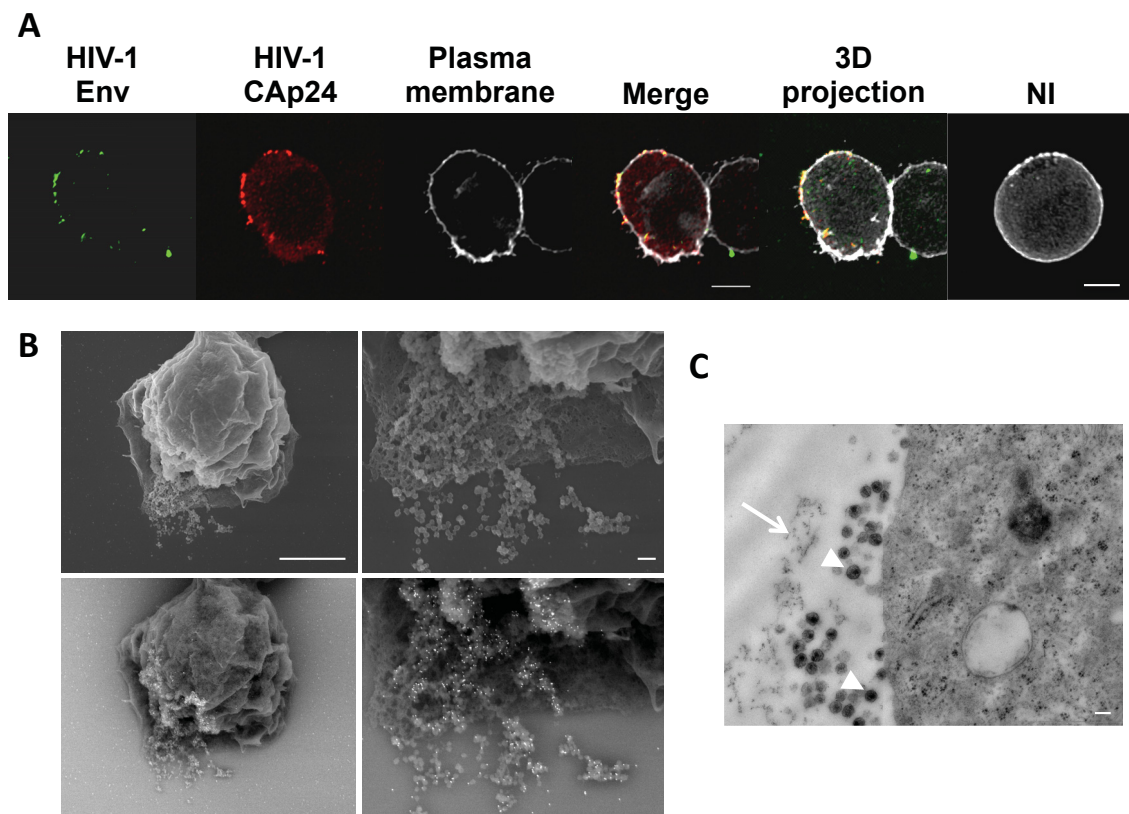


**Figure 22. HIV-1 infected T cells carry extracellular mature viral material.**

*Confocal fluorescence imaging of J1.1 cells, ACH2 cells, primary CD4<sup>+</sup> T cell infected with HIV-1 NL4-3, BaL and DH12, and primary CD4<sup>+</sup> T cells from HIV-1 patients showing aggregates of HIV-1 material (red) at the surface of the plasma membrane (green). HIV-1 is stained using an anti-MAP17 antibody except for J1.1 cells and BaL for which an anti-CAP24 antibody was used. Plasma membrane is stained using anti-CD98 antibody for cell lines J1.1 and ACH2 and using anti-CD45 for primary CD4<sup>+</sup> T cells. Scale bars: 5  $\mu$ m.*

Confocal imaging revealed that HIV-1 viral material accumulating at T cell surface is positive for HIV-1 CAp24 and MAp17 (Figure 22) as well as for Env glycoprotein staining (Figure 23A). Scanning electron microscopy depicted this extracellular mature viral material as aggregates of round viral particles staining positive for anti-Env immunogold labeling (Figure 23B). Furthermore, transmission electron microscopy showed that these viral particles have a classical dense-core morphology of mature viral particles (Figure 23C).

HIV-1 surface-bound viral material is hence composed of aggregates of Env+ mature dense-core viral particles. Interestingly, those clusters of viral particles were surrounded by an electron-dense material (Figure 23C) that was strongly reminiscent of the morphology of ECM proteins enriched in HTLV-1 biofilm.

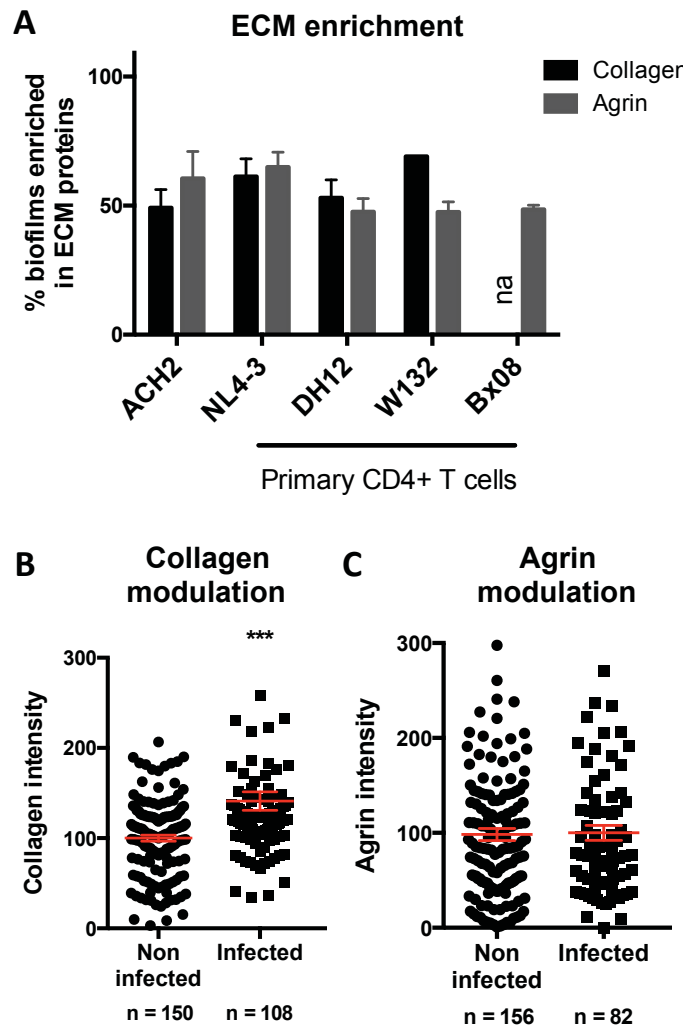


**Figure 23. HIV-1 extracellular material is composed of Env+ dense-core viral particles.**

*A. Confocal fluorescence image of a primary CD4<sup>+</sup> T cell infected with HIV-1 NL4-3. Non-permeabilized anti-Env immunostaining (green) accumulates in aggregates of viral material (anti-CAp24, red) at the surface of the plasma membrane (anti-CD45, gray). NI = Non Infected, Scale bars: 5 µm. B. Scanning Electron Micrographs of HIV-1 material at the surface of J1.1 cells. Extracellular aggregates of spherical viral particles of 100 nm in diameter stain positive for anti-Env immunogold staining. Scale bars: 1 µm left, 100 nm right. C. Transmission Electron Micrographs of HIV-1 viral particles at the surface of J1.1 cells. Aggregates of dense core-mature viral particles of 100 nm in diameter (arrowheads) are embedded in an electron-dense material (arrow). Scale bar: 100 nm.*

### 3. HIV-1 infected T cells produce a viral biofilm

We next sought to characterize some components enriched in HIV-1 extracellular aggregates. As HIV-1 infection is known to modulate the expression of a broad range of host proteins, and among them some ECM proteins, we searched whether this electron-dense phase is composed of ECM proteins. We brought a particular attention to collagen, reported to be over-expressed upon HIV-1 infection (Smith et al., 2010; Vigneault et al., 2011), and to the heparan sulfate proteoglycan agrin expressed by T cells in physiological conditions (Khan et al., 2001). As conventional collagen labeling using antibodies requires methanol post-fixation that deeply alters the morphology of the cells, we favored the use of the versatile probe Ace-A1488 that enables a specific collagen labeling in native conditions prior to PFA fixation (Zong et al., 2005). Collagen and agrin were consistently found enriched in HIV-1 biofilms, as 49 to 69% and 47 to 65% of HIV-1 extracellular aggregates were enriched in collagen and agrin resp., depending on the cell type and virus considered (Figure 24A). Interestingly, and as previously reported in other permissive cell types (Smith et al., 2010), we found collagen expression by CD4+ T cells to be upregulated by HIV-1 infection (Figure 24B) while the expression of agrin appeared globally unaffected by HIV-1 infection (Figure 24C).

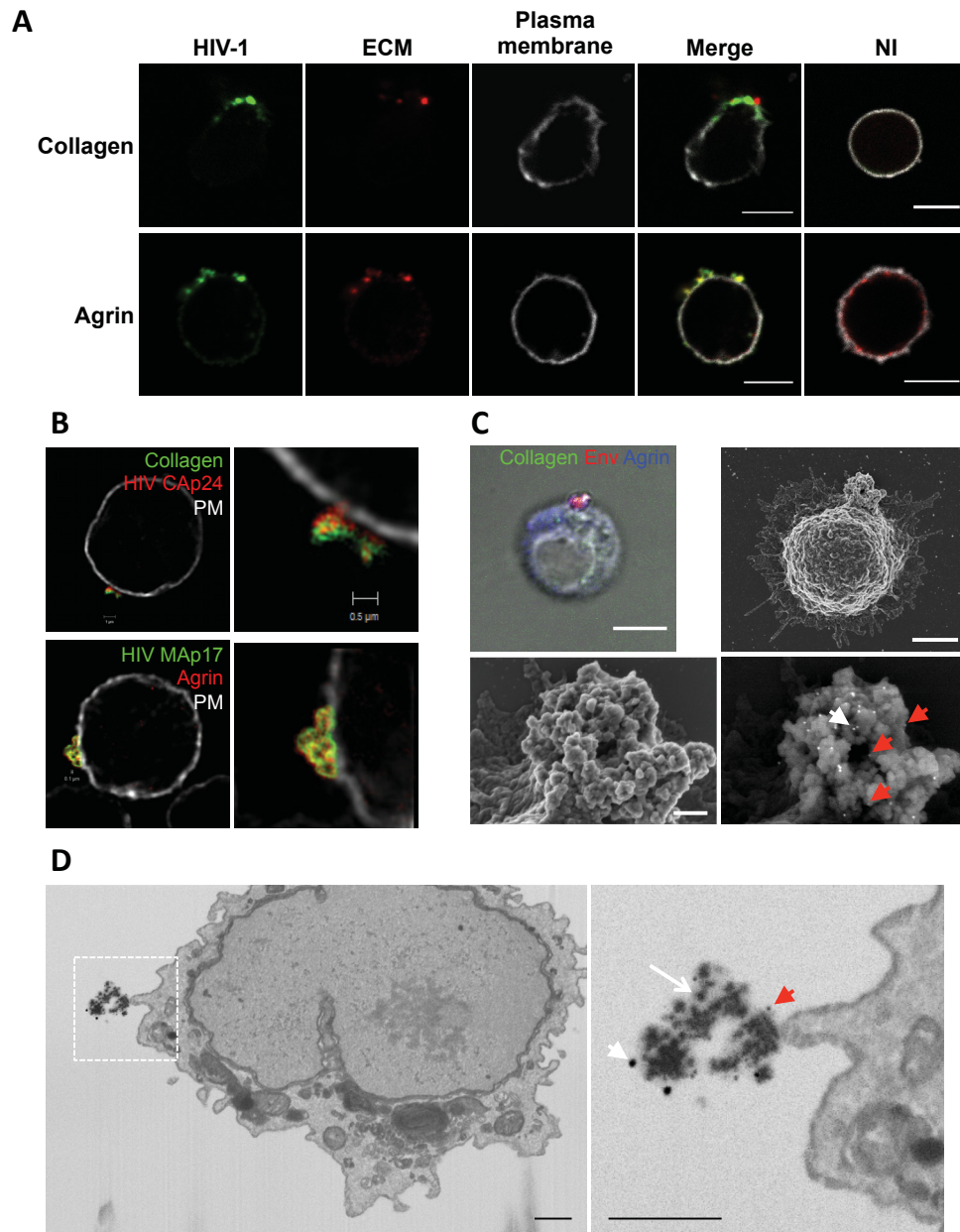


**Figure 24. Collagen and agrin accumulation in HIV-1 biofilm and modulation upon HIV-1 infection.**

*A. Quantification of the fraction of cells carrying viral extracellular aggregates enriched in collagen (black) and agrin (gray) for ACH2 cells and primary CD4+ T cells infected with HIV-1 laboratory strains NL4-3 and BaL and virus primary isolates DH12, W132 and Bx08, means + SEM of at least 150 cells from 3 independent experiments. na = not available. (B-C) Primary CD4+ T cells were infected with HIV-1 DH12, surface-stained for collagen or agrin and stained in permeabilizing conditions for HIV-1 CAp24 or MAp17. Shown are intensity values quantified on confocal images for infected and corresponding non infected cells from the same donor. **B.** Quantification of collagen intensity for infected CAp24+ cells. **C.** Quantification of agrin intensity for infected MAp17+ cells.*

Confocal microscopy on CD4<sup>+</sup> T cells from HIV-1 patients revealed HIV-1 extracellular aggregates they carry to be specifically enriched in collagen and agrin as well (Figure 25A). Structured Illumination Microscopy allowed a more precise description of the organization of those two ECM proteins within HIV-1 biofilm (Figure 25B). Agrin hence appeared to individually surround each viral particle whereas collagen rather formed a cap coating aggregates of several viral particles. Correlative confocal/Scanning Electron Microscopy shows that HIV-1 biofilms from HIV-1 patients can be as large as one tenth of the volume of the cell and stain positive for collagen in fluorescence and for agrin and HIV-1 Env both in fluorescence and immunogold labeling (Figure 25C). FIB-SEM further unveiled the organization of HIV-1 biofilm, staining positive for both anti-HIV-1 Env and anti-Agrin immunogold labelings (Figure 25D). In addition, FIB-SEM revealed that for HIV-1 patients as well, HIV-1 biofilm is composed of dense-core mature viral particles.

As viral biofilms are defined as extracellular aggregates of viral particles enriched in ECM proteins (Pais-Correia et al., 2010), it can thus be stated that, similar to HTLV-1, HIV-1 infected T cells form a viral biofilm attached to the surface of the infected cell.



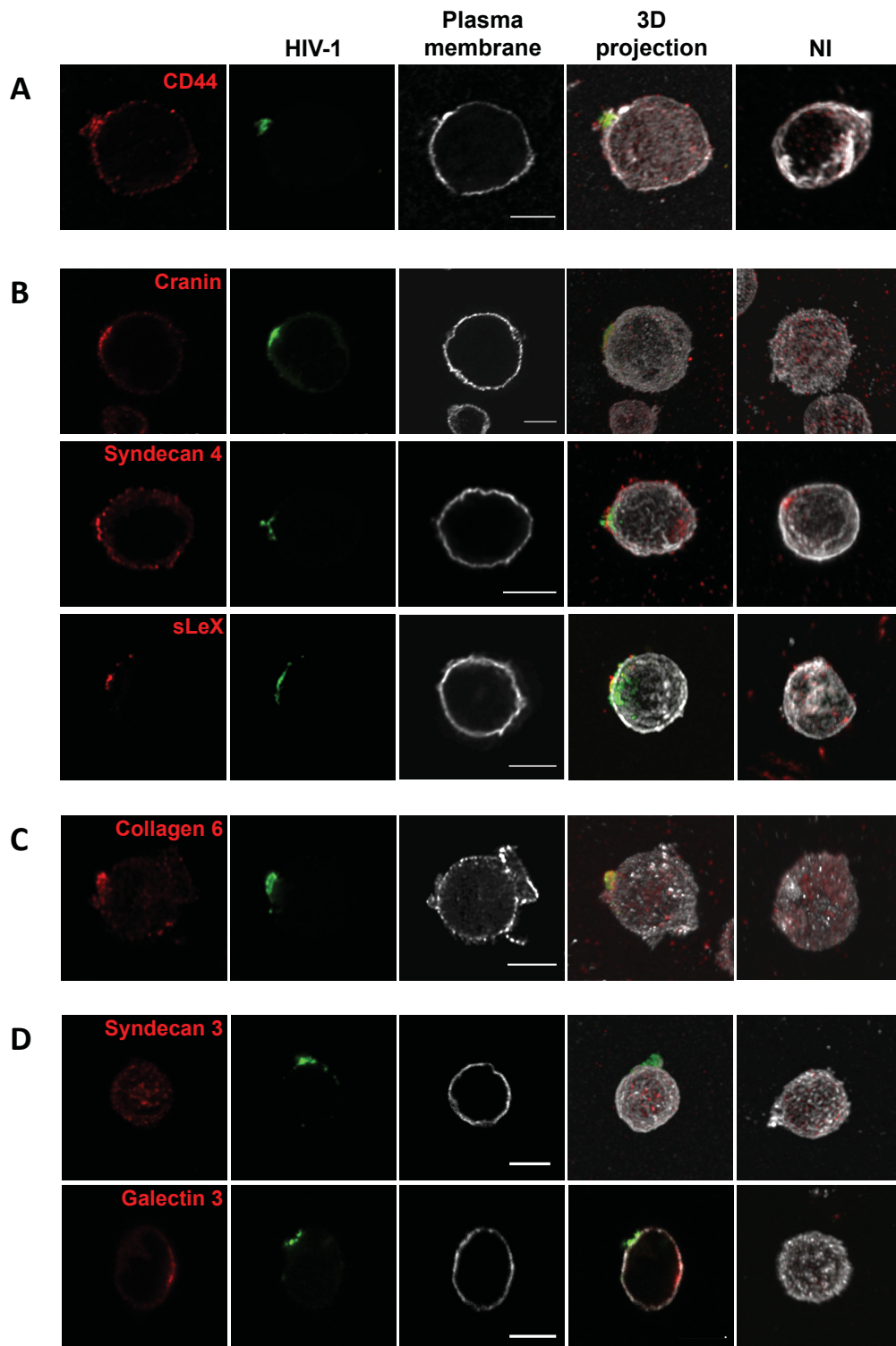
**Figure 25. HIV-1 extracellular aggregates form a biofilm enriched in collagen and agrin.**

Primary CD4<sup>+</sup> T cells from HIV-1 patients were observed using complementary imaging techniques. **A.** Confocal fluorescence images showing collagen (top panel) and agrin (bottom panel) in red and HIV-1 viral material (CAp24 top panel, MAp17 bottom panel) in green at the surface of the plasma membrane (CD45, gray). NI = non infected CD4<sup>+</sup> T cells. Scale bars: 5  $\mu$ m. **B.** Structured Illumination Microscopy showing collagen (top panel) and agrin (bottom panel) in green and HIV-1 viral particles in red at the surface of the plasma membrane (CD45, gray). Scale bars: 1  $\mu$ m. **C.** Correlative Confocal-Scanning Electron microscopy. Top left panel: confocal fluorescence image showing HIV-1 Env (red), collagen (green), and agrin (blue) immunostainings overlaid with transmission image. Scale bar: 5  $\mu$ m. Top right panel: Scanning Electron micrograph of the same cell. Scale bar: 2  $\mu$ m. Bottom left panel: enlargement. Scale bar: 300 nm. Bottom right panel: HIV-1 Env (10 nm, red arrowheads) and agrin (20 nm, white arrowhead) immunogold labelings. **D.** Thin sections of a Focused Ion Beam-Scanning Electron Microscopy (FIB-SEM) acquisition of HIV-1 biofilm displaying dense-core viral particles (arrow) staining positive for HIV-1 Env (10 nm, red arrowhead) and surrounded by Agrin (20 nm, white arrowhead). Scale bars: 500 nm.

A deeper characterization of HIV-1 biofilm components by immunofluorescence revealed that HIV-1 biofilm is enriched in syndecan 4, upregulated upon HIV-1 infection (Vigneault et al., 2011), cranin, a glycoprotein displaying agrin-binding capacities (Gee et al., 1994), as well as in collagen 6 (Figure 26A&B). The carbohydrate moiety sialyl Lewis X, involved in lymphocyte adhesion processes (Munro et al., 1992), was found enriched in HIV-1 biofilm as well (Figure 26A). On the contrary, syndecan 3 and galectin 3 were clearly excluded from HIV-1 biofilm (Figure 26C).

Interestingly, HIV-1 biofilm was also enriched in the transmembrane heparan-sulfate CD44 (Figure 26D), known to accumulate at the uropod of T cells (del Pozo et al., 1997), to incorporate within HIV-1 viral particles (Bastiani et al., 1997; Bregnard et al., 2013; Chertova et al., 2006; Stephenson et al., 2010) and to interact with a broad range of ECM ligands (Ishii et al., 1993; Jalkanen and Jalkanen, 1992; Lesley and Hyman, 1992).

HIV-1 biofilm is thus enriched in specific ECM components, some of them, such as collagen, being modulated by HIV-1 infection.



**Figure 26. ECM components accumulated or absent from HIV-1 biofilm.**

Confocal imaging of primary CD4<sup>+</sup> T cells infected with HIV-1 primary isolates DH12 or Bx08 (**A**, **B**, **D**) or of ACH2 cells (**C**) show HIV-1 biofilm containing viral material (Map17 or Cap24, green) at the surface of the plasma membrane (CD45, gray). **A**. HIV-1 biofilm is enriched in CD44 (red). **B**. HIV-1 biofilm is enriched in Cranin, Syndecan 4 and sLeX (red). **C**. HIV-1 biofilm is enriched in Collagen 6. The plasma membrane is stained using anti-CD98 Ab. **D**. Syndecan 3 and Galectin 3 (red) are excluded from HIV-1 biofilm. Non infected cells are shown as controls. Scale bars: 5  $\mu$ m.



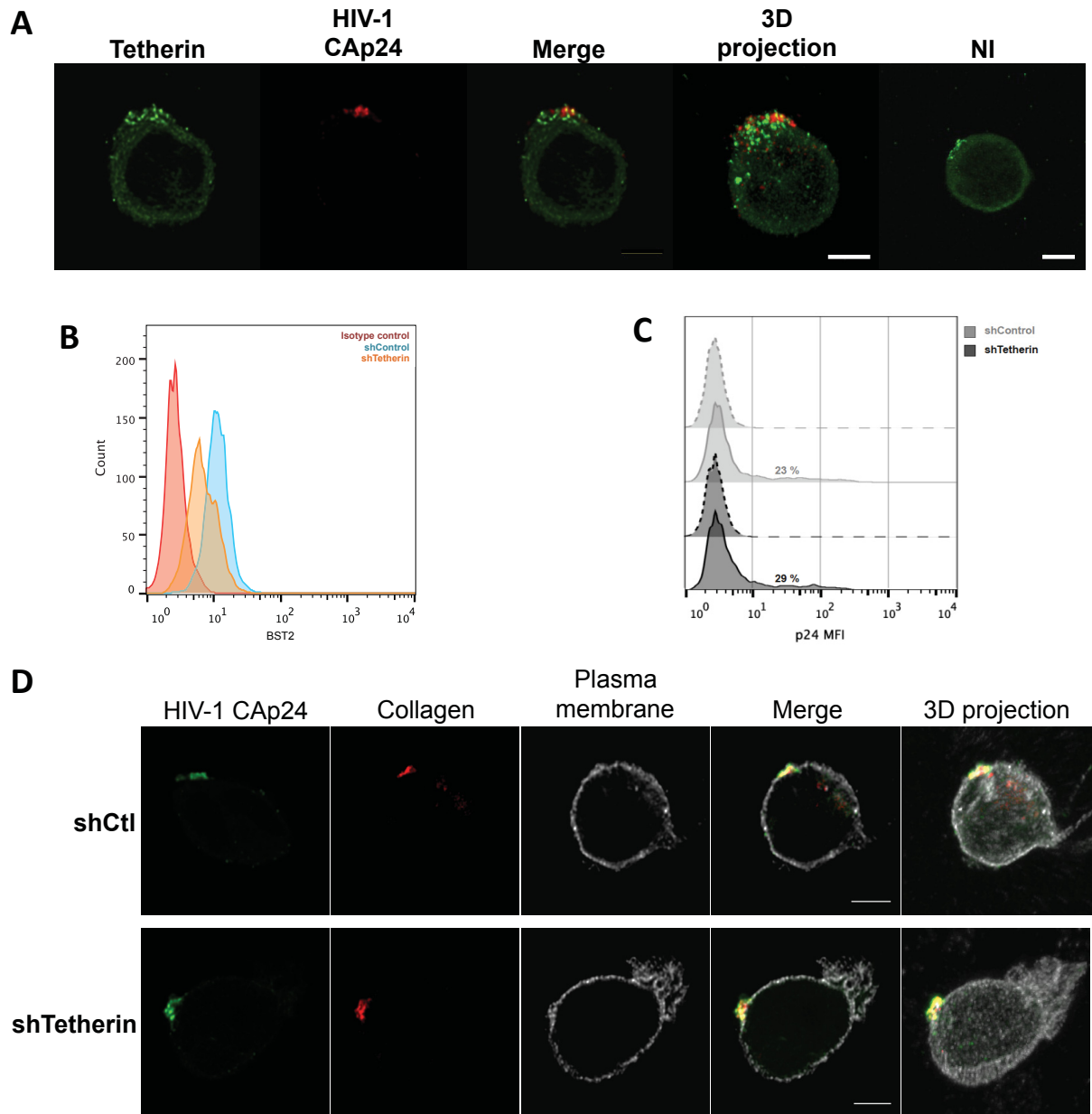
#### 4. Tetherin is a component of HIV-1 biofilm

IFN-inducible host membrane protein Tetherin retains virions at the surface of HIV-1 infected cells (Hammonds et al., 2010; Perez-Caballero et al., 2009; Sauter et al., 2010); yet viral accessory protein Vpu triggers the degradation of Tetherin (Neil et al., 2008), thus favoring the release of cell-free viral particles. Surprisingly, Tetherin was consistently found accumulated in biofilms formed by primary CD4<sup>+</sup> T cells infected with all HIV-1 WT viruses tested (shown for virus primary isolate Bx08 (Figure 27A)). In order to assess the contribution of Tetherin in HIV-1 biofilm formation, we used a shTetherin CEM T cell line (Casartelli et al., 2010) in which Tetherin silencing was of 60% as compared to corresponding shControl CEM (Figure 27B). We infected this shTetherin cell line with HIV-1 NL4-3 and monitored biofilm formation. It is worth to note that the results presented here arise from only 2 experiments. Infection of shTetherin cells is currently reproduced for confirmation and quantification.

According to these first series of data, at similar infection rates of shControl and shTetherin cells (Figure 27C), only 49% of infected CAp24<sup>+</sup> shTetherin cells carried a biofilm as compared to the 63% measured for shControl cells. However, when produced by shTetherin cells, HIV-1 biofilm appeared to have a wild-type morphology with a classical size, albeit a little smaller, and the usual enrichment in collagen (Figure 27D). Even, fraction of CAp24 aggregates enriched in collagen was higher for the shTetherin cell line, with 81% of collagen+CAp24<sup>+</sup> aggregates for only 67% in the shControl cell line.

Although a reduced proportion of shTetherin cells is able to form a biofilm, frequency of collagen<sup>+</sup> biofilms is higher for shTetherin cells, indicating that in the absence of Tetherin, collagen might take over to retain viral particles at the cell surface. Tetherin thus represents one component of HIV-1 biofilm participating in the retention of viral particles at the cell surface; yet Tetherin is not strictly required for HIV-1 biofilm formation.

***HIV-1 T-cell associated infectivity thus mostly resides in a viral biofilm retained and exposed at the surface of T cells and enriched in Tetherin and ECM components.***



**Figure 27. Tetherin is enriched in HIV-1 biofilm yet is not required for its formation.**

*A. Confocal fluorescence image of a primary CD4<sup>+</sup> T cell infected with virus primary isolate Bx08. Tetherin is shown in green and HIV-1 CAp24 in red. Scale bar: 5  $\mu$ m. B. Flow cytometry analysis of total Tetherin expression in shControl (blue) and shTetherin (orange) CEM cells. Corresponding isotype control is shown in pink. (C-D) Results are representative of two experiments. C. Flow cytometry analysis of CAp24 expression for shControl (light gray) and shTetherin (dark gray) CEM cells. Non infected cells are shown in dashed lines, fraction of CAp24<sup>+</sup> cells is indicated above the histograms. D. Confocal fluorescence images showing HIV-1 biofilm composed of viral material (CAp24, red) and collagen (green) at the surface of the plasma membrane (CD98, gray) for both shControl and shTetherin cells. Scale bars: 5  $\mu$ m.*

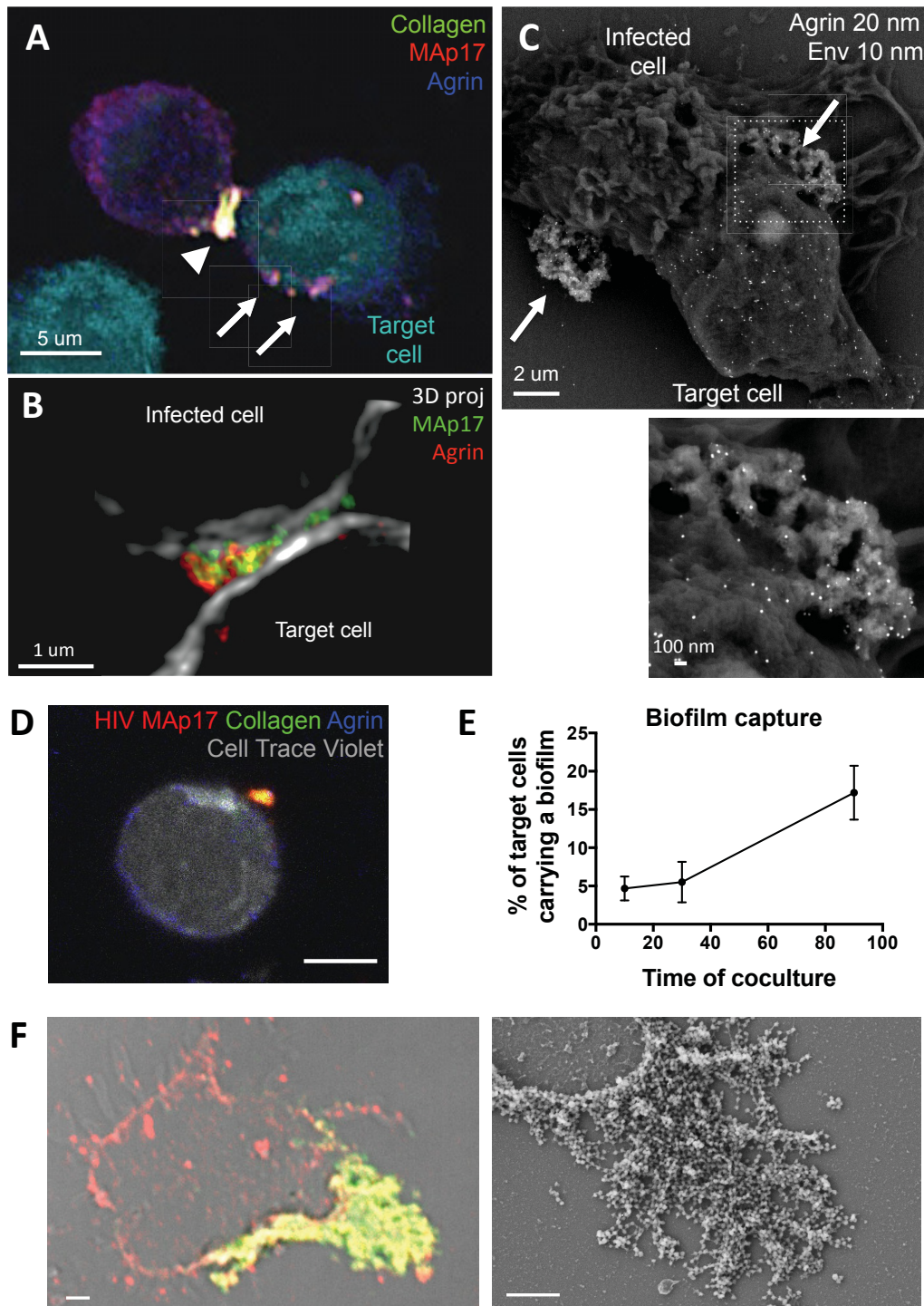
## **II. HIV-1 biofilm mediates direct and indirect cell-to-cell spread**

### **1. HIV-1 biofilm is transferred at the virological synapse along with its ECM components**

In order to gain more insight into the participation of HIV-1 biofilm ECM components in cell-associated infectivity, we monitored the fate of HIV-1 biofilm during cell-to-cell transfer. Confocal imaging of virological synapses between ACH2 and CEM cells revealed that HIV-1 biofilm is transferred at the virological synapse as a cohesive entity, as agrin and collagen were transferred together with HIV-1 MAP17 to non-infected target T cells (Figure 28A). Structured Illumination Microscopy of a synapse spontaneously forming between an infected and a non-infected cell from an HIV-1 patient showed an intimate interaction between HIV-1 virions and ECM components upon transfer at the intercellular junction (Figure 28B). Similarly, Scanning Electron Micrograph of a similar junction highlighted the preserved cohesion of HIV-1 biofilm upon transfer between T cells, as both agrin and HIV-1 Env immunogold labeling appeared enriched in transferred biofilm (Figure 28C). As HIV-1 biofilm was accumulating at the interface between infected and target cells in 55 % of the junctions studied, adhesive properties of highly glycosylated ECM components enriched in HIV-1 biofilm might act as glue stabilizing intercellular junctions.

Further, adhesive properties of HIV-1 biofilm enabled the capture and retention of viral material at the surface of target T cells along with biofilm ECM components (Figure 28D). Transfer of HIV-1 biofilm between T cells even appeared as a rather efficient mean of dissemination as already 5% of target T cells carry a biofilm after 10 min of co-culture with biofilm-producing cells, reaching about 20% after 90 min of co-culture (Figure 28E). HIV-1 biofilm hence enabled a massive transfer of viral particles to target T cells. Highly adhesive properties of HIV-1 biofilm were further confirmed by its adsorption on an inert surface formed by a polylysine-coated glass coverslip and subsequent dissociation of the biofilm-producing cell (Figure 28F). Scanning Electron Micrograph revealed the large amount of viral particles contained in HIV-1 biofilm and their apparent organization in networks along fibers.

HIV-1 biofilm hence represents a highly adhesive structure enabling the efficient and massive transfer of viral particles to target cells.



**Figure 28. HIV-1 biofilm is a highly adhesive structure transferred as a cohesive entity at the virological synapse.**

*A. Confocal fluorescence imaging of biofilm transfer between an infected ACH2 cell and a target CEM cell (CellTrace Violet, cyan). Biofilm stains positive for HIV-1 MAp17 (red), collagen (green) and agrin (blue). Scale bar: 5  $\mu\text{m}$ . B. Structured Illumination Microscopy of biofilm transfer between an infected T cell and a non-infected T cell from an HIV-1 patient. Biofilm stains positive for HIV-1 MAp17 (green) and Agrin (red) at the surface of the plasma membrane (gray). Scale bar: 1  $\mu\text{m}$ . C. Scanning electron micrograph showing biofilm transfer between an infected T cell and a non-infected T cell from an HIV-1 patient. Agrin (20 nm) and Env immunogold stainings (10 nm) accumulate in the*

biofilm. Scale bar: 2  $\mu\text{m}$ . Inset: scale bar 100 nm. **D.** Confocal fluorescence imaging of a target CEM cell (Cell Trace Violet, gray) carrying HIV-1 biofilm, that stains positive for HIV-1 MAp17 (red), collagen (green) and agrin (blue). Scale bar: 5  $\mu\text{m}$ . **E.** Quantification of the fraction of target CEM cells carrying a biofilm after 10, 30 or 90 min of coculture with ACH2 cells. **F.** Correlative Confocal-Scanning Electron Micrograph of HIV-1 biofilm from ACH2 cells adsorbed on a glass coverslip. HIV-1 Env: green, agrin: red, scale bars: 1  $\mu\text{m}$ . SIM was performed by Marina Caillet and SEM and FIB/SEM were performed by Perrine Bomme.

## 2. ECM components of HIV-1 biofilm are crucial for HIV-1 infectivity

As ECM components are transferred together with viral material upon formation of T-T junctions, we next sought to determine the properties conferred by ECM components to HIV-1 transmission. Since collagen is over-expressed by HIV-1 infected cells (Smith et al., 2010; Vigneault et al., 2011) and accumulated in HIV-1 biofilm, we wanted to assess the role collagen plays in HIV-1 transmission. We therefore subjected HIV-1 biofilm produced at the surface of ACH2 cells to collagenase digestion. A range of concentrations of *Clostridium* collagenase D generated up to 20% reduction in cell-associated MAp17, as assessed by flow cytometry (Figure 29A). Decrease in cell-associated MAp17 correlated to a reduced cell-associated infectivity (Figure 29B). In addition, confocal imaging revealed that collagenase D disorganized HIV-1 biofilm, yielding a dotted pattern of small viral aggregates scattered all over the surface of infected T cells (Figure 29C). Importantly, collagenase treatment did not affect cell viability (Figure 29D). Although measured only once for the moment, infectivity of ultracentrifuged cell culture supernatant did not seem to be altered by collagenase treatment (Figure 29E), further supporting the prominent participation of biofilm collagen in HIV-1 retention and organization at T cell surface and subsequent cell-to-cell transmission.

As the heparan sulfate agrin was found enriched in HIV-1 biofilm, we next challenged the participation of biofilm heparan sulfates to HIV-1 transmission using the heparan sulfate competitor heparin. Addition of heparin during mechanical washes did not further affect cell-associated CAp24 as measured by flow cytometry (Figure 29F). Nonetheless, heparin washes induced a decrease in cell-associated infectivity of 25% as compared to mechanical washes alone (Figure 29G). Reduced HIV-1 transmission was potentially linked HIV-1 biofilm reorganization, as heparin treatment appeared to reduce the thickness of surface-bound viral aggregates, spreading them in thinner but expanded clusters at the surface of the plasma membrane (Figure 29H). Through organization of viral material at the cell surface, heparan sulfates enriched in HIV-1 biofilm thus contribute to the efficiency of HIV-1 transmission between T cells.

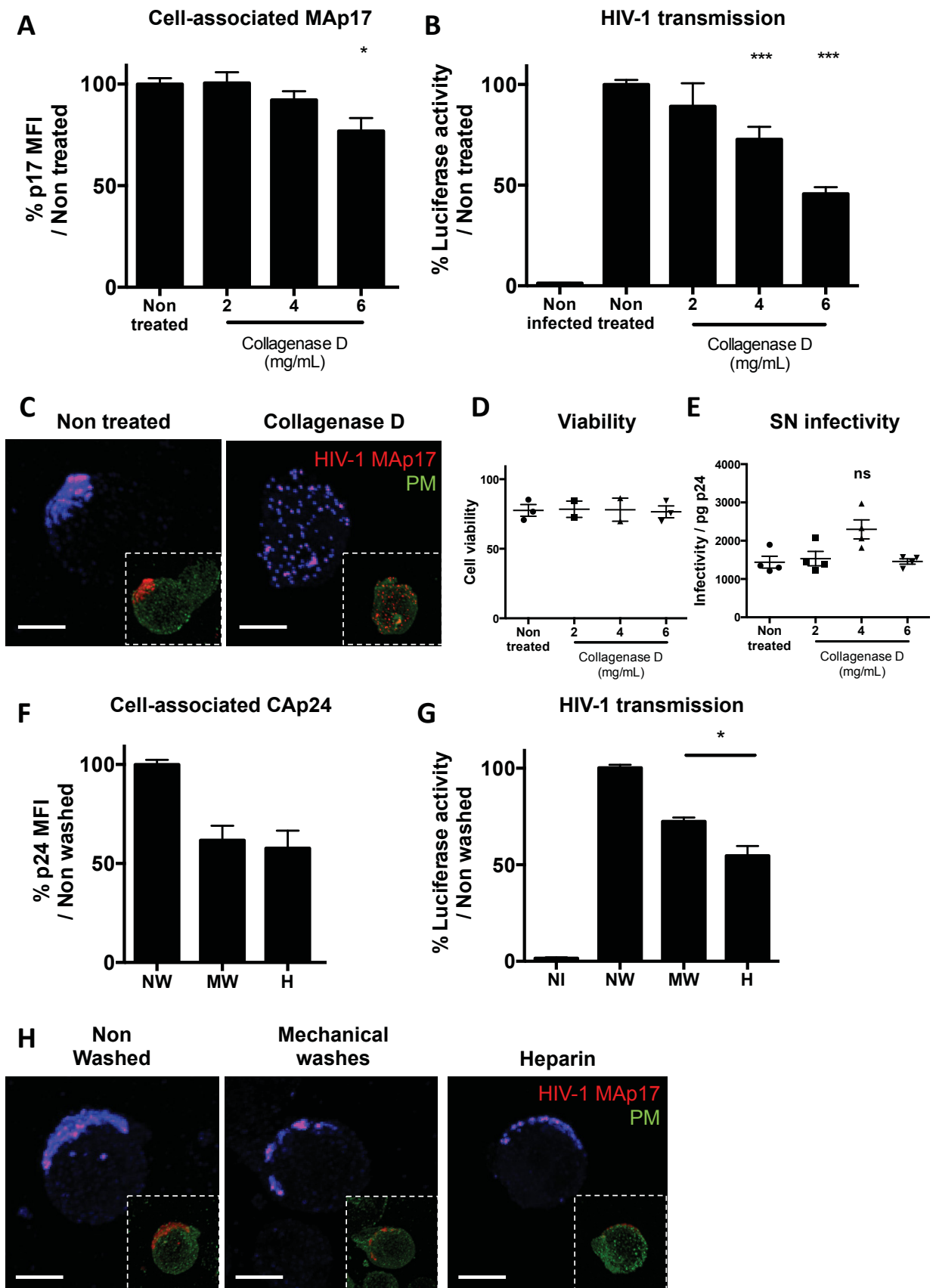
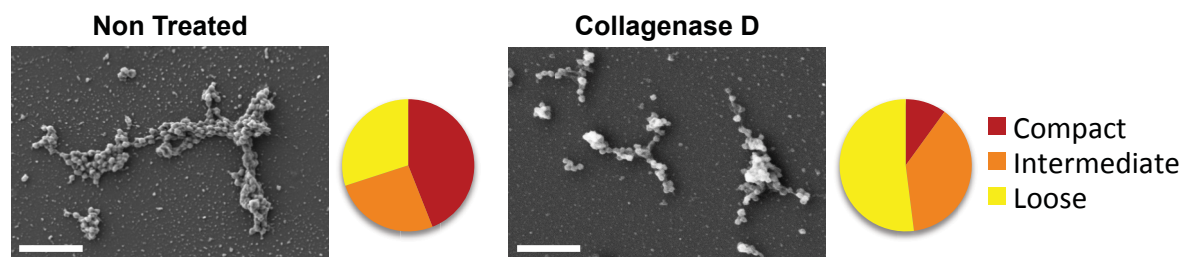


Figure 29. Biofilm collagen and heparan sulfates are crucial for HIV-1 infectivity.

**(A-E)** ACH2 cells were treated overnight with a range of Collagenase D doses. **(F-H)** ACH2 cells were left non-washed (NW) or mechanically washed in the absence (MW) or presence of Heparin (H). **A, F.** Flow cytometry quantification of cell-associated MAp17 (**A**) or CAp24 (**F**). MFI: Mean Fluorescence Intensity. **B, G.** Luciferase assay determining cell-associated infectivity. Non-infected cells (NI) are used as controls. **C, H.** Confocal imaging of cell-associated MAp17 (ultra or red) at the surface of the plasma membrane (CD98, green). Scale bars: 5  $\mu$ m. **D.** Fraction of viable cells measured by flow cytometry (FSC+SSC+). **E.** Infectivity of cell culture supernatants (SN) after ultracentrifugation removing collagenase enzyme, normalized by the amount of CAp24. Shown are triplicates from one experiment. Unless stated otherwise, shown are means + SEM of triplicates from three independent experiments. *P*-values are calculated using Mann-Whitney tests (\*:  $p < 0.1$ , \*\*\*:  $p < 0.001$ ).

Collagenase digestion thus led to the formation of a dotted pattern of small viral aggregates at the surface of biofilm-producing cells. Further, collagenase treatment of biofilms adsorbed on a glass coverslip seemed to loosen fibers otherwise clustering viral particles together (Figure 30C). Should this result be confirmed, collagen thus contributes to the efficiency of HIV-1 transmission not only by retaining viral particles at the cell surface but also by conferring HIV-1 biofilm with a specific organized architecture.

ECM biofilm components, and especially heparan sulfates and collagen, are thus crucial for HIV-1 cell-associated infectivity.



**Figure 30. Collagenase D treatment unpacks HIV-1 viral biofilm.**

*Scanning Electron Micrographs of viral biofilms from ACH2 cells adsorbed on polylysine-coated coverslips and left Non treated or digested for 6 h with 10 mg/mL of Collagenase D, and corresponding quantifications of Compact, Intermediate and Loose biofilms measured for 50 biofilms. Scale bars: 1  $\mu$ m. Shown are values for one experiment.*

### 3. HIV-1 biofilm participates in DC-mediated *trans*-infection

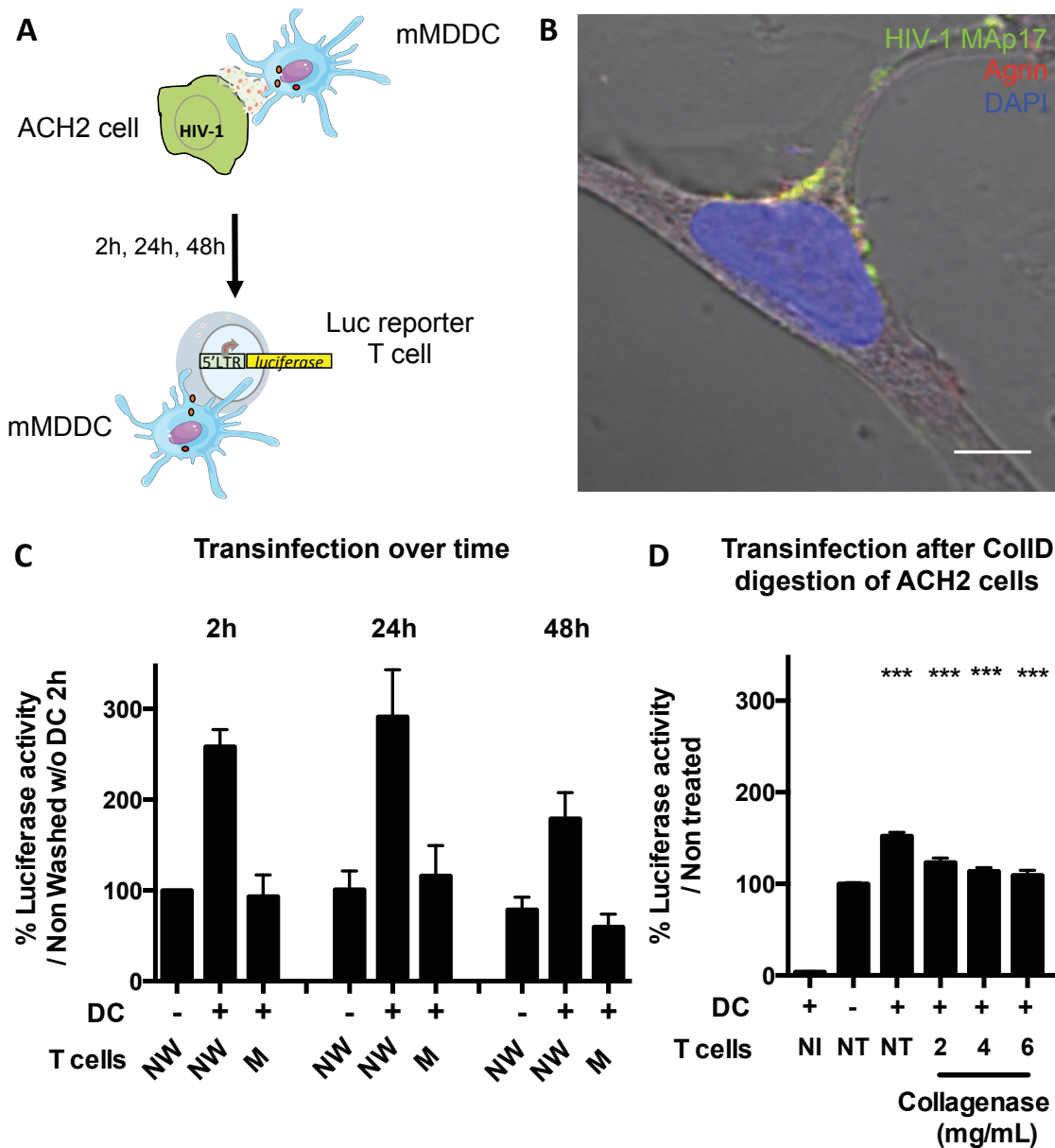
HIV-1 cell-to-cell transmission can be either direct between T cells across a virological synapse or indirect, involving the capture of virions by poorly permissive cells such as mature dendritic cells (DCs) and their transmission to target T cells in a process called DC-mediated *trans*-infection (Cameron et al., 1992). Mature DCs display reduced phagocytic capacity, a poor permissivity to HIV-1 infection and are more efficient at mediating *trans*-infection than their immature counterparts (Cavrois et al., 2007). As viral synapses are reported to encompass the transfer of clustered virions from DCs to T cells (McDonald et al., 2003), we evaluated the participation of viral clusters forming HIV-1 biofilm in the *trans*-infection process.

We therefore set-up a “tripartite system” involving the co-culture of biofilm-producing ACH2 cells with primary mature monocyte-derived dendritic cells (mMDDCs) followed by subsequent addition of Luciferase reporter T cells (Figure 31A). We observed that mMDDCs captured virions pre-clustered in HIV-1 biofilm together with ECM proteins such as agrin (Figure 31B). Co-culture with mMDDCs led to an efficient *trans*-infection effect in our experimental conditions, as coculture of biofilm-producing non-washed T cells with mMDDCs yielded a 4-fold increase in infectivity as compared to direct T-T transmission (Figure 31C). As in T-T transfer, cell-associated infectivity mostly originated from T cell surface since mechanical washes of ACH2 cells abrogated infectivity by 4-fold in the context of *trans*-infection (Figure 31C). In addition, biofilm effect appeared as potent as the *trans*-infection effect, as both absence of mMDDCs or biofilm mechanical removal led to a 4-fold decrease in HIV-1 transmission (Figure 31C).

Contribution of HIV-1 biofilm to *trans*-infection efficiency was further challenged through collagenase pre-treatment of ACH2 biofilm-producing cells. While *trans*-infection efficiency peaks at 24h of DC-ACH2 coculture (Figure 31C), we purposely measured the effect of collagenase digestion after only 2h of DC-ACH2 coculture in order to avert the masking effect of *de novo* collagen synthesis that could occur upon 24h of coculture. Importantly, collagenase digestion reduced the efficiency of *trans*-infection, further supporting the prominent role played by HIV-1 biofilm in the efficiency of *trans*-infection (Figure 31D). HIV-1 biofilm can thus be captured by mMDDCs and transferred in *trans* to target T cells, thus contributing to the efficiency of the *trans*-infection process.

***HIV-1 biofilm thus efficiently mediates HIV-1 direct and indirect cell-to-cell spread and biofilm ECM components condition the efficiency of this transmission.***





**Figure 31. HIV-1 biofilm is captured and transferred *in trans* by mature dendritic cells.**

**A.** Schematic representation of the experimental set-up. Biofilm-producing ACH2 cells are cocultured with mature MDDCs and luciferase reporter T cells are subsequently added to the coculture at indicated time-points. **B.** Confocal imaging of a mMDDC having captured HIV-1 biofilm staining positive for HIV-1 MAp17 (green) and agrin (red) after 2h of coculture with ACH2 cells. Fluorescence imaging is overlaid with transmission signal. **C.** Luciferase assay determining the infectivity of Non Washed (NW) and Mechanically Washed (M) ACH2 T cells in the presence (+) or absence (-) of mMDDCs. Reporter T cells were added after 2, 24 or 48h of DC-ACH2 coculture as indicated. Results are normalized by infectivity of Non Washed cells in the absence of DCs at 2h, shown are means + SEM of triplicates from 4 independent experiments each performed with mMDDCs from at least two independent donors. **D.** Luciferase assay determining cell-associated infectivity of Non Infected (NI), Non Treated (NT) or collagenase D-digested ACH2 T cells in the presence (+) or absence (-) of mMDDCs (DC). Reporter T cells were added after 2h of DC-ACH2 coculture. Results are normalized by infectivity of Non treated cells in the absence of DCs, shown are means + SEM of triplicates from two independent experiments each performed with mMDDCs from two independent donors. P-values are calculated using Mann-Whitney tests (\*:  $p < 0.1$ , \*\*\*:  $p < 0.001$ ).

### III. HIV-1 biofilm increases viral infectivity

#### 1. Viral particles within HIV-1 biofilm are more infectious than their cell-free counterparts

While HTLV-1 disseminates exclusively across intercellular contacts, HIV-1 transmission involves both the formation of intercellular junctions and the dissemination of cell-free viral particles. Despite this major discrepancy, both HTLV-1 (Pais-Correia et al., 2010) and HIV-1 infected T cells produce a biofilm. HIV-1 “bimodal transmission” enables the comparison of the relative infectivity of cell-free versus cell-associated viral particles accumulated within HIV-1 biofilm. To this end, we mechanically washed biofilm-producing ACH2 cells, quantified by ELISA the amount of CAp24 released by mechanical washes  $Q$  and complemented biofilm-depleted mechanically washed cells with 1.5  $Q$  of cell-free CAp24 collected from culture supernatants (Figure 32A). This complementation in cell-free viruses did not rescue the infectivity of biofilm-carrying non-washed T cells both in direct LT-LT transmission (Figure 32B) and in the context of *trans*-infection (Figure 32C), indicating that viral particles accumulated in HIV-1 biofilm at the surface of the producing cell are more infectious than their cell-free counterparts. Interestingly, infectivity of free-virus was increased in the presence of mechanically washed cells but also, to a lesser extent, in the presence of non infected cells, both in the presence and absence of mMDDCs (Figure 32D).

In order to assess whether this increase in viral particle infectivity is an intrinsic property of HIV-1 biofilm independent of the carrying cell, we compared the infectivity of detached biofilms collected from rinses of mechanical washes to the infectivity of free viral particles collected from corresponding cell-culture supernatants. Scanning Electron Micrographs showed that isolated biofilms are composed of dozens of viral particles embedded in a mesh of ECM components whereas cell culture supernatants are mainly composed of isolated viral particles (Figure 32E). When normalized by the amount of CAp24 protein, viral particles accumulated within the viral biofilm display a 6-fold increase in infectivity as compared to cell-free viral particles (Figure 32F).

Increased infectivity of HIV-1 viral particles within HIV-1 biofilm hence appears as an intrinsic feature of HIV-1 biofilm independent of its exposure at the surface of the infected producing T cell.

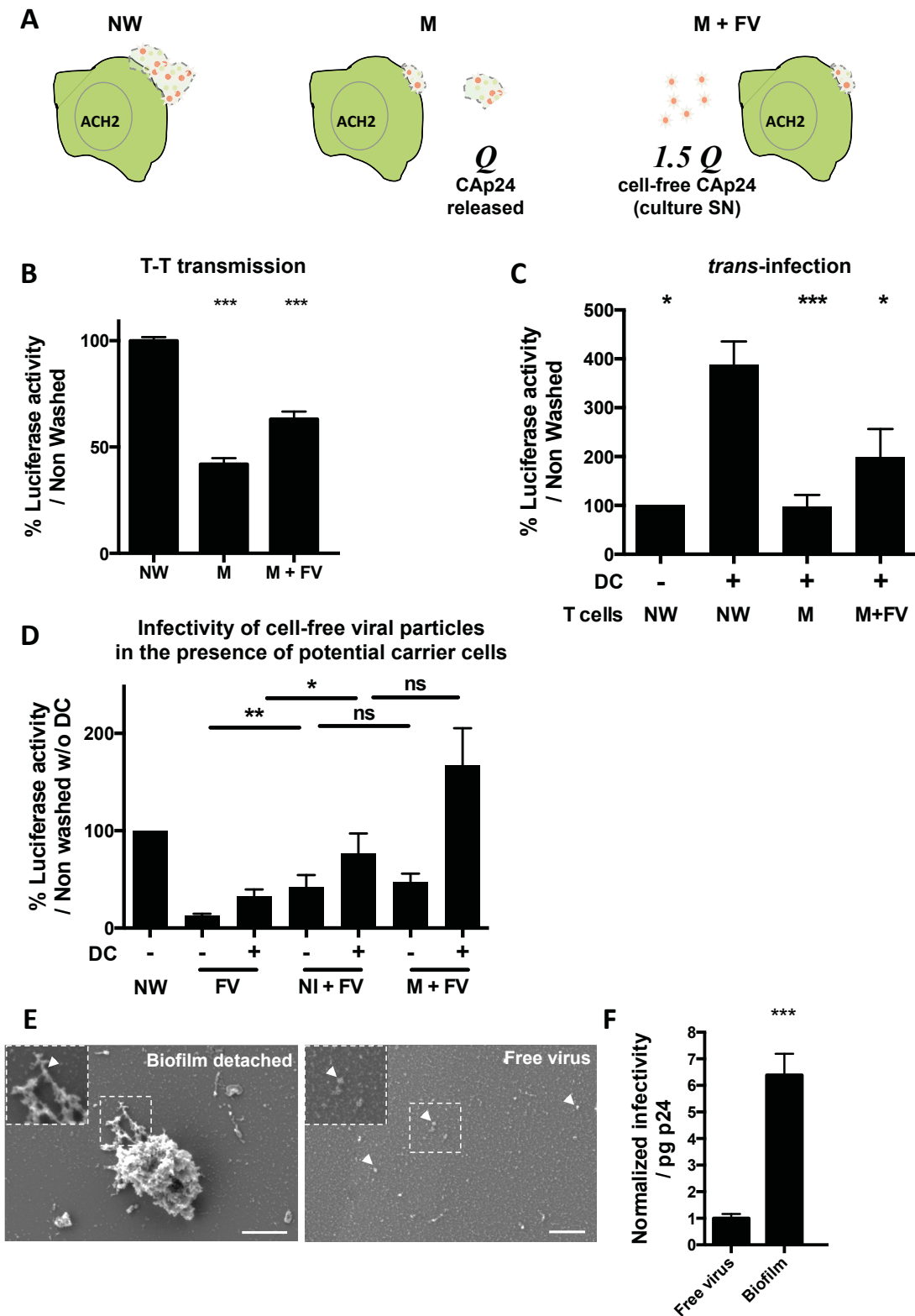


Figure 32. Viral material is more infectious within HIV-1 biofilm than equal amount of free viral particles.

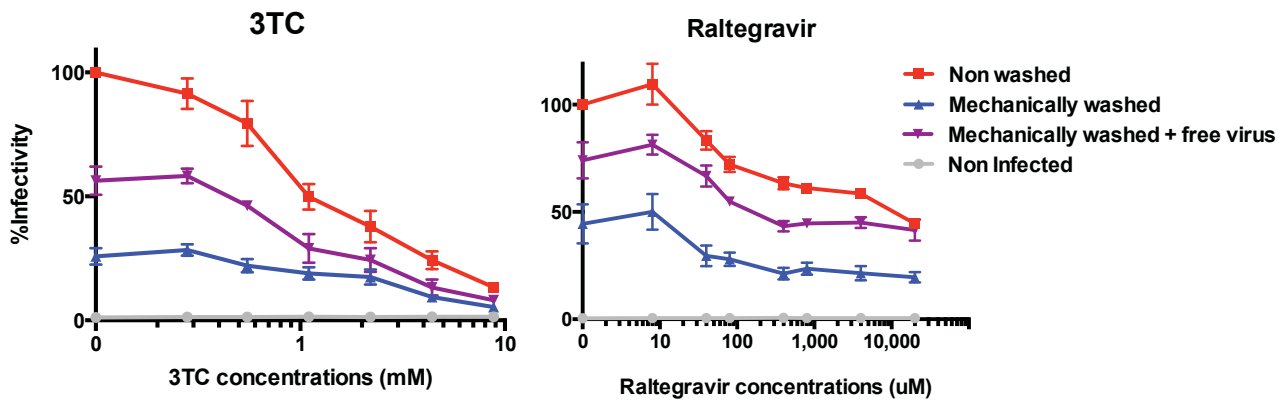
*A. Schematic representation of the experimental set-up. Biofilm-producing ACH2 cells left non washed or subjected to mechanical washes. Amount of CAp24 released by mechanical washes  $Q$  is measured by ELISA and mechanically washed cells are complemented with  $1.5 Q$  the amount of cell-free viral particles collected from corresponding cell culture supernatant (SN). B. Luciferase assay on Jurkat LTR-luciferase clone determining the infectivity of non washed cells (NW), mechanically washed cells (M) and mechanically washed cells complemented with cell-free viral particles (M+FV), mean + SEM of triplicates from 12 independent experiments. C. Luciferase assay determining the infectivity of non washed cells (NW), mechanically washed cells (M) and mechanically washed cells complemented with cell-free viral particles (M+FV), in the presence (+) or absence (-) of mMDDCs, mean + SEM of triplicates from 4 independent experiments each performed with mMDDCs from at least two donors. D. Luciferase assay determining the infectivity of free viral particles (FV), non infected cells complemented with cell-free viral particles (NI+FV) and mechanically washed cells complemented with cell-free viral particles (M+FV), in the presence (+) or absence (-) of mMDDCs, mean + SEM of triplicates from 4 independent experiments each performed with mMDDCs from at least two donors. E. Scanning Electron Micrograph of mechanically detached biofilm and free virus from ACH2 cells. Scale bars: 1  $\mu$ m. F. Luciferase assay determining the infectivity of cell-free viral particles and detached biofilm normalized by the amount of CAp24, mean + SEM of triplicates from four independent experiments.*

## **2. HIV-1 biofilm reduces the sensitivity to antiretroviral treatment (ArT)**

Cell-associated infectivity is reported to reduce HIV-1 sensitivity to some molecules used in ArT (Agosto et al., 2014; Gupta et al., 1989; Sigal et al., 2011; Titanji et al., 2013; Zong et al., 2005). In order to assess the contribution of HIV-1 biofilm to this reduced sensitivity, we compared the infectivity of biofilm-producing non-washed ACH2 cells in the presence of ArT to the infectivity of mechanically washed cells complemented or not with free viral particles. Increasing doses of nucleoside analog reverse-transcriptase inhibitor 3TC and integration inhibitor Raltegravir were unable to abolish the increased infectivity conferred by HIV-1 biofilm. No matter the ArT concentration, non-washed cells indeed displayed infectivity 1.2- to 1.8-fold higher than mechanically washed cells complemented with cell-free viral particles and at least 2-fold higher than mechanically washed cells (Figure 33).

Increased infectivity conferred by HIV-1 biofilm was thus preserved at ArT concentrations impairing cell-free infection. In other words, when 1 mM of 3TC is required to abrogate the infectivity of biofilm-producing cells by 50%, 5 times less 3TC (0.2 mM) is required to reach the same infectivity for mechanically washed cells complemented with free virus.

By increasing viral particle infectivity, HIV-1 biofilm hence contributes to the reduced sensitivity to ArT observed for cell-associated infectivity.



**Figure 33. Biofilm-mediated infectivity reduces HIV-1 sensitivity to ArT.**

*Luciferase assay determining the infectivity of Non Infected cells (gray), Non Washed cells (red), Mechanically Washed cells (blue) and Mechanically washed cells complemented with cell-free viral particles (purple) in the presence of a range of concentrations of 3TC and Raltegravir, means + SEM of triplicates from 3 independent experiments.*

### 3. HIV-1 biofilm reduces the sensitivity to neutralizing antibodies

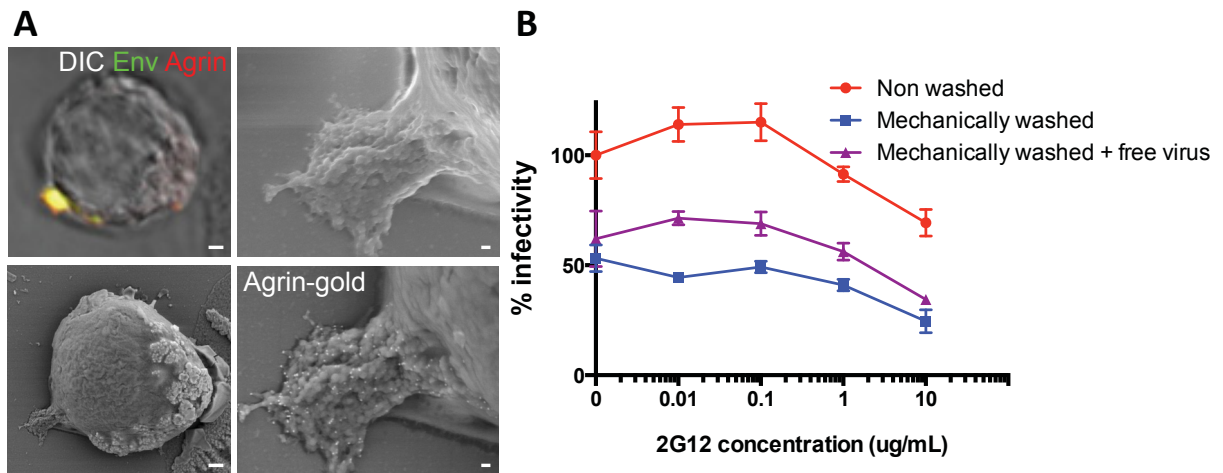
HIV-1 cell-associated infectivity is reported to display reduced sensitivity to some neutralizing antibodies (Chen et al., 2007; Gupta et al., 1989; Malbec et al., 2013; Schiffner et al., 2013). We therefore wanted to assess the contribution of HIV-1 biofilm to this protection from NABs.

So far, we performed SEM observations of HIV-1 biofilm at room temperature following dehydration of the sample that might have altered the structure of ECM highly glycosylated proteins enriched in HIV-1 biofilm. In order to observe biofilm ECM in hydrated conditions, we applied a cryo-SEM technique conventionally used to study bacterial biofilms to the observation of HIV-1 biofilm. Thus preserved, HIV-1 biofilm appeared as a protective microenvironment in which ECM glycosylated components surround viral particles in a “jelly-like” shell (Figure 34A). Successfully combined to correlative Confocal/SEM, this cryo-SEM technique will be soon transposed to the characterization of biofilms from HIV-1 patients. Appendix 4 shows the draft of the method article currently in preparation to describe this technique.

In order to challenge the contribution of HIV-1 biofilm microenvironment to the reduced sensitivity to NABs, we incubated biofilm-producing ACH2 cells with NAb 2G12. Although requiring further confirmation, our preliminary data indicate that increasing doses of NAb 2G12 were inefficient at abrogating biofilm-mediated infectivity of non-washed ACH2 cells (Figure 34B). Hence, no matter the concentration of 2G12, biofilm-mediated

infectivity remained at least 2.1 fold higher than infectivity of mechanically washed cells and 1.6 fold higher than infectivity of mechanically washed cells complemented with cell-free viral particles (Figure 34B).

Overall, advantage in infectivity conferred to viral particles by HIV-1 biofilm is preserved at 2G12 concentrations otherwise impairing cell-free infection. HIV-1 biofilm thus contributes to the reduced sensitivity to NAbs observed for HIV-1 cell-associated infectivity.



**Figure 34. HIV-1 biofilm is a protective microenvironment reducing the sensitivity to 2G12.**

*A. Cryo-correlative Confocal/Scanning Electron Microscopy of HIV-1 biofilm staining positive for HIV-1 Env (green) and agrin (red, 20 nm). Scale bars left: 1 um, right: 100 nm. B. Luciferase assay determining the infectivity of Non Infected cells (gray), Non Washed cells (red), Mechanically Washed cells (blue) and Mechanically washed cells complemented with cell-free viral particles (purple) in the presence of increasing concentrations of NAb 2G12, means + SEM of triplicates from 2 independent experiments.*

***Biofilm-associated HIV-1 virions are thus more infectious than their cell-free counterparts and this increased infectivity is preserved in the presence of ArT and NAbs.***

## **In conclusion,**

The present work shows that most of T-cell associated infectivity resides at the cell surface. T-cell associated infectivity indeed relies on the accumulation of mature dense-core Env<sup>+</sup> viral particles at the surface of HIV-1 infected T cells. Our study identifies those extracellular viral aggregates as viral biofilms in which extracellular matrix components are accumulated in a regulated and apparently organized manner. More specifically, collagen and agrin, are consistently found enriched in HIV-1 biofilm. Although not required for biofilm formation, Tetherin also participates in the retention of HIV-1 virions at the cell surface.

Production of HIV-1 biofilm was evidenced for every infected T cells studied, namely HIV-1 chronically infected T cell lines, primary CD4<sup>+</sup> T cells infected *in vitro* with both HIV-1 laboratory strains and virus primary isolates and, importantly, for CD4<sup>+</sup> T cells from HIV-1 patients.

Biofilm ECM components are modulated by HIV-1 infection; for instance, collagen is upregulated whereas the heparin sulfate agrin is reorganized at the cell surface. We show that both collagen and heparan sulfates enriched in HIV-1 biofilm have a crucial contribution to viral particle retention and organization at the cell surface as well as to HIV-1 intercellular transmission. HIV-1 biofilm is indeed transferred as a cohesive entity both in the context of direct T-T transmission as well as in the *trans*-infection process and ECM components contribute to the efficiency of this transmission.

HIV-1 biofilm provides HIV-1 transmission with several features. Mainly, HIV-1 biofilm increases the infectivity of HIV-1 virions, either exposed at the cell surface or attached to inert surfaces. In addition, HIV-1 biofilm participates in the reduced sensitivity to ArT and NAbs characterizing HIV-1 transmission by cell contacts.

Overall, the present work unravels the existence of a new extracellular viral pool with specific properties that have a major contribution to the efficiency of HIV-1 intercellular transmission.

**80%**

of T-cell associated infectivity is due to HIV-1 biofilm

Exposed at T cell surface

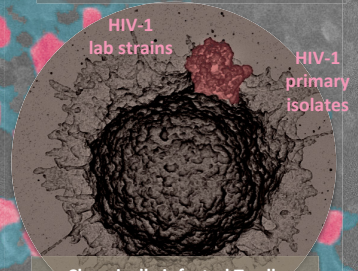
Crucial for HIV-1 infectivity

**ECM components**

Collagen, agrin, syndecan 4, cranin, sLEX

Protective microenvironment?

Highly adhesive structure



Chronically infected T cells  
Primary CD4+ T cells  
CD4+ T cells from HIV-1 patients

**Efficient transmission**

T-T, DC-mediated *trans*-infection, inert surface, reduced sensitivity to ArT and NABs

More infectious than cell-free virions

Locally increased infectious titer

**Dozens of viral particles**





## **MATERIAL AND METHODS**



## ***Cells***

Peripheral blood mononuclear cells from healthy donors or from HIV-1 patients were isolated through Ficoll-Hypaque centrifugation.

CD4<sup>+</sup> T cells were isolated by negative selection with magnetic cell sorting (Miltenyi Biotec, MACS) and CD4<sup>+</sup>-enriched T cell population was cultured in AIM-V medium complemented with 40 U/mL IL-2 (Chiron) and 5 ng/mL IL-7 (R&D systems).

CD14<sup>+</sup> cells were isolated by positive selection with magnetic cell sorting (Miltenyi Biotec, MACS). CD14<sup>+</sup> cells were then differentiated into MDDC through culture for 5 days in AIM-V medium in the presence of 100 U/mL GM-CSF (Miltenyi Biotec) and 20 ng/mL IL-4 (Miltenyi Biotec). MDDC were matured through 24 h of culture in the presence of 25 ug/mL poly-IC (Sigma) and 5 ng/mL TNF- $\alpha$  (R&D systems).

Blood samples from HIV-1 patients beginning Highly Active Antiretroviral Treatment (HAART) were obtained in the context of the Biomedical Research Program TRANSBioHIV promoted by the French National Agency for AIDS and Hepatitis (ANRS). All individuals gave informed consent. Clinical characteristics of study subjects are shown in Appendix 3.

HIV-1 chronically infected T cell line ACH2 and its parental cell line CEM were a kind gift from Pr. U.Hazan. Reporter cells TZMbl and HIV-1 chronically infected T cell line J1.1 were obtained through the NIH AIDS Reagent Program. CEM shControl and shTetherin were a kind gift from Dr N.Casartelli (Casartelli et al., 2010). Cells lines were cultured in RPMI-1640 (Gibco) containing 10% FCS, 10 IU/mL penicillin and 10  $\mu$ g/mL streptomycin and selection agent hygromycine (400 ug/mL) or puromycine (0.5 ug/mL) when required.

## ***Viruses and infections***

HIV-1 virus primary isolates Bx08, DH12 and W132 were obtained through the NIH AIDS Reagent Program. For non-peusotyped viruses, CD4<sup>+</sup> T cells (10<sup>6</sup> cells/mL) were infected at 20 to 45 ng of p24/10<sup>6</sup> cells in 96-U-well plates using a spinoculation protocol. Infections with VSV-G pseudotyped viruses were performed through incubation for 3 h with primary CD4<sup>+</sup> T cells. Infected cells were used for further experiments after 3 to 5 days, when the infection rate reached 20 to 45 %.

## ***Reagents***

Rabbit antibodies (Ab) to agrin (707T and 204) were gifts from Dr M. Rüegg. Rabbit anti-HIV-1 CAp24 Ab and human neutralizing Ab to HIV-1 gp120 2G12 were obtained through the NIH AIDS Reagent Program. Mouse IgG1 mAb to HIV-1 MAb17 (18-A), to CAp24 (25-A) and to HIV-1 gp120 (110H) were from Hybridolabs, Pasteur. Mouse IgG2a mAb to CD45 (GAP 3,8) was home-made. Mouse IgG2a mAb to CD98 was from R&D Systems. Polyclonal mouse anti-Cranin Ab was from Chemicon. Polyclonal mouse anti-Collagen 6 Ab was from Calbiochem. Mouse polyclonal anti-Tetherin Ab and rabbit anti-Syndecan 4 and anti-Galectin 3 Ab were from Abcam. Rabbit anti-Syndecan 3 Ab was from Atlas. Mouse IgM anti-sLeX, mouse IgG1 anti-fibronectin, mouse IgG2b anti-CD44 mAbs and FITC-conjugated anti-p24 Ab KC57 were from Becton-Dickinson. DAPI was from Invitrogen. FITC-coupled Ab to mouse IgG1, IgG2a and IgM were from Southern Biotechnology. Cy3-coupled Ab to rabbit IgG and to mouse IgG1, IgG2b and IgG as well as Alexa647-coupled Ab to mouse IgG2a were from Jackson ImmunoResearch. Cy5-coupled Ab to rabbit Ig was from GE Healthcare. Annexin V/PI, Cell Trace Violet and Alexa488-coupled Ab to fluorescein was from Life technologies. 20 nm colloidal gold protein A was from the University Medical Center Utrecht, 10 nm colloidal gold anti-mouse IgG+IgM Ab was from British Biocell International. The plasmid encoding firefly Luciferase under the control of HIV-1 Long Terminal Repeat (pLTR-Luc) was a kind gift from Dr. S. Emiliani (Reynolds et al., 2003).

## ***Collagen labeling***

Versatile collagen probe Ace was produced in a bacterial system using a plasmid given by Dr. M. Hook (Zong et al., 2005) and subsequently coupled to Alexa488. Collagen was labeled through overnight incubation of the cell culture with Ace in serum-free medium. Production and optimization of the labeling conditions were performed by Marina Caillet.

## ***Detachment of extracellular viral assemblies from infected cells***

HIV-1-infected cells were left unwashed, rinsed once to remove virions loosely adhering to the surface of the cells or submitted to one to four rounds of extensive pipeting followed by a rinse in RPMI-1640 serum-containing medium. Alternatively, one or two rounds of extensive pipetings were performed in Heparin-containing medium (50 ug/mL, Sigma) and cells were gently rinsed three times in order to remove contaminating Heparin. Cells were

then processed for immunofluorescence analysis, flow cytometry and co-culture with a reporter cell line. Viral antigens were detected in cell culture supernatants or detachment supernatants by Gag p24 ELISA according to the manufacturer's instructions (Zeptometrix). When indicated, Mechanically washed cells were complemented with Free Virus collected from cell culture supernatants. The amount of p24 added corresponded to 1.5 fold the amount of p24 detached from the Mechanically washed cells, as assessed by p24 ELISA.

### ***Collagenase D and Subtilisin treatments***

Infected cells were treated with Subtilisin (5 mg/mL, Sigma) or Collagenase D (2, 4 or 6 mg/mL, Roche) at 37°C in serum-containing medium, for 5 min or 12 h resp. Subtilisin treatment was stopped by adding an excess of Protease Inhibitor (Roche). Cells were gently rinsed three times in order to remove contaminating enzymes.

### ***Luciferase reporter gene assay***

Jurkat J77c120 cells were transfected with the plasmid pLTR-Luc described above. These luciferase reporter T cells were cultured in a 96-well round-bottom plate with HIV-1–infected cells at a cell ratio of 1:1 and 3:1 for infected cell lines and primary infected CD4<sup>+</sup> T cells resp. After 20 or 30 h resp, we assessed luciferase activity using a Promega luciferase kit assay and a TR717 Microplate Luminometer (Berthold Technologies).

### ***Coculture assays***

Target non-infected CEM cells were pre-loaded with CellTrace Violet (Invitrogen) and cocultured at a ratio 1:1 with biofilm-producing ACH2 cells prestained with the Ace anti-collagen probe. Cocultures were subsequently fixed in an equivolume of PFA 8 % at 10, 30 and 90 min of coculture.

### ***Antiretroviral treatment and neutralizing antibody assays***

Luciferase reporter T cells were preincubated for 1h with increasing concentrations of antiretroviral treatments (ArT) 3TC and Raltegravir prior to coculture with HIV-1–infected cells in the presence of ArT. Conversely, HIV-1 infected cells were preincubated for 1h with increasing concentrations of 2G12 NAb prior to coculture with the luciferase reporter T cell line in the presence of 2G12.

### ***Adhesion assay***

Cells were loaded with CFSE (Invitrogen) and incubated for 40 min in wells coated with fibronectin (R&D systems). After several washes, adhering cells were lysed and fluorescence was measured using a Tecan fluorimeter.

### ***Flow cytometry***

Cells were fixed with 4 % paraformaldehyde for 10 min at 25 °C and Gag p17 or p24 were stained using 18-A (Hybridolabs, Pasteur) or KC57 FITC (Becton-Dickinson) resp. through incubations in solutions containing 0.05 % saponin. Cells were analyzed on a BD Bioscience FacsCalibur.

### ***Immunofluorescence, confocal microscopy and Structured Illumination Microscopy***

For fluorescence imaging, cells were fixed with 4 % paraformaldehyde for 10 min at 25°C. Surface staining was performed in the absence of detergent. Intracellular proteins and Gag p17 or p24 were stained through incubations in solutions containing 0.05 % saponin. DAPI was added in the last staining step. Confocal microscopy analysis was carried out on upright and inverted Zeiss LSM700 using 40× and 63× objectives. For illustration, Z series of optical sections were acquired at 0.2 μm increments and images were deconvoluted using Huygens software. Structured Illumination Microscopy was carried out by Marina Caillet on a Zeiss LSM780 ELYRA system using a 63× objective.

### ***Correlative Confocal/ Scanning Electron Microscopy***

Following overnight incubation with the collagen probe Ace, cells were fixed on alphanumeric coded, grid-patterned glass (MatTek dishes) with 4% paraformaldehyde for 10 min at 25°C. Surface staining was performed in the absence of detergent with primary antibodies targeting HIV-1 Env and agrin followed by secondary anti-mouse IgG1 coupled to Cy3 and anti-mouse coupled to 10 nm gold to detect Env, and anti-rabbit coupled to Cy5 and protein A gold 20 nm to detect agrin. Cells were first imaged by confocal microscopy as described above, then fixed by immersion in 2.5 % glutaraldehyde, and post-fixed with 1 % OsO<sub>4</sub>. Subsequently, samples were dehydrated through increasing graded ethanol series and subjected to critical point drying using Carbon Dioxide (CO<sub>2</sub>) as transitional medium. Glass coverslip were mounted on a specimen stub using colloidal silver paint (Electron Microscopy

Sciences) and sputter coated with 2 nm of carbon. SEM imaging was carried out with a ZEISS Auriga Crossbeam system (Carl Zeiss NTS) using a secondary electron (SE2) detector. Immunogold labeling was detected using back-scattered electron detector (YAG) on a Jeol JSM 6700F microscope with a field emission gun operating at 5 kV.

### ***Correlative Confocal/Focused Ion Beam-Scanning Electron Microscopy (FIB-SEM)***

#### *Sample preparation*

Cells were first imaged by confocal microscopy on an alphanumeric coded, grid-patterned glass (MatTek dishes). Cells were processed from adapted OTO protocol (Seligman et al., 1966). Briefly, infected cells were fixed by immersion in 2.5 % glutaraldehyde, and post-fixed. First in a mix of 1 % OsO<sub>4</sub> with 1.5 % K<sub>4</sub>Fe(CN)<sub>6</sub> (potassium ferrocyanide – reduced osmium) to increase membrane contrast, then contrast was enhanced by 1 % tannic acid. A second post-fixation was performed in 1 % OsO<sub>4</sub>. Subsequently, samples were dehydrated through increasing graded ethanol series and embedded in epoxy resin (Electron Microscopy Sciences) at 60°C for 48 h. The embedded resin block was mounted on a specimen stub using colloidal silver paint (Electron Microscopy Sciences) and sputter coated with 200 nm of gold/palladium.

#### *FIB/SEM image stack acquisition*

Focused ion beam milling and SEM imaging were carried out with a ZEISS Auriga Crossbeam system (Carl Zeiss NTS) equipped with Atlas3D (Fibics Inc., Ottawa) (Heymann et al., 2009; Murphy et al., 2011). A focused ion beam (30 kV, 500 pA) iteratively removed slices (thickness: 10 nm for the biofilm attached to the cell and 5 nm for the biofilm detached). The scanning electron beam was used to record images at pixel sizes of 5 nm in the xy plane. The SEM images were recorded using an energy selective back-scattered electron (ESB) detector.

#### *Image processing and analysis*

Individual 2D images were merged, cropped, and aligned using customized scripts based on the image processing programs ImageJ (Girish and Vijayalakshmi, 2004) and ICY (de Chaumont et al., 2012). Output images were then loaded into Amira 5.0.1 (Visage Imaging, Berlin, Germany) software package. Structures of interest were manually segmented and reconstructed.





## **DISCUSSION AND PERSPECTIVES**



# **I. HIV-1 T cell-associated infectivity relies on cell-surface viral particles retained in a viral biofilm**

## **1. Surface-bound virions are crucial for T-cell associated infectivity**

CD4<sup>+</sup> T cells are the major replication sites for HIV-1 and viral transmission from infected to non-infected T cells through intercellular contacts represents a crucial mean of dissemination (Chen et al., 2007; Dimitrov et al., 1993). Characterizing the nature of the viral material transferred at cellular junctions is of prime importance in understanding the modalities of HIV-1 transmission between T cells. In DC-mediated *trans*-infection, viral material retained at the surface of DCs in membrane invaginations is the main source of infectivity. By contrast, the participation of membrane-associated virions to HIV-1 transmission between T cells still remains an open question.

The present work evidences HIV-1 surface-bound viral particles as the major contributors to HIV-1 T-cell infectivity. Mechanical removal of surface-associated HIV-1 material reduces HIV-1 T to T transmission by 40 to 80% (Figure 20). Of note, the contribution of surface-bound virions to T-cell associated infectivity might partially be underestimated due to *de novo* production of surface-bound and cell-free viral particles during the contact with the LTR-reporter cell line. Ideally, the absolute contribution of HIV-1 surface-bound virions could have been accurately evaluated in the presence of an inhibitor of HIV-1 assembly or budding (Lemke et al., 2012) that is so far not commercially available.

Microscopic analyses identify HIV-1 surface-bound material as a viral biofilm containing mature dense-core Env<sup>+</sup> viral particles (Figure 23) and enriched in some extracellular matrix components (Figure 25&Figure 26). This biofilm can be efficiently captured and transferred to non-infected target T cells upon formation of intercellular contacts (Figure 28). Viral particles are already known for long to be retained and accumulated at the surface of infected T cells. So far, this retention has been mainly attributed to Tetherin and considered to be characteristic of viruses defective for viral accessory protein Vpu (Hammonds et al., 2010; Neil et al., 2008; Perez-Caballero et al., 2009; Sauter et al., 2010). Implication of factors other than Tetherin for viral retention was not explored nor characterized. Careful analysis of microscopic studies of the virological synapse published by

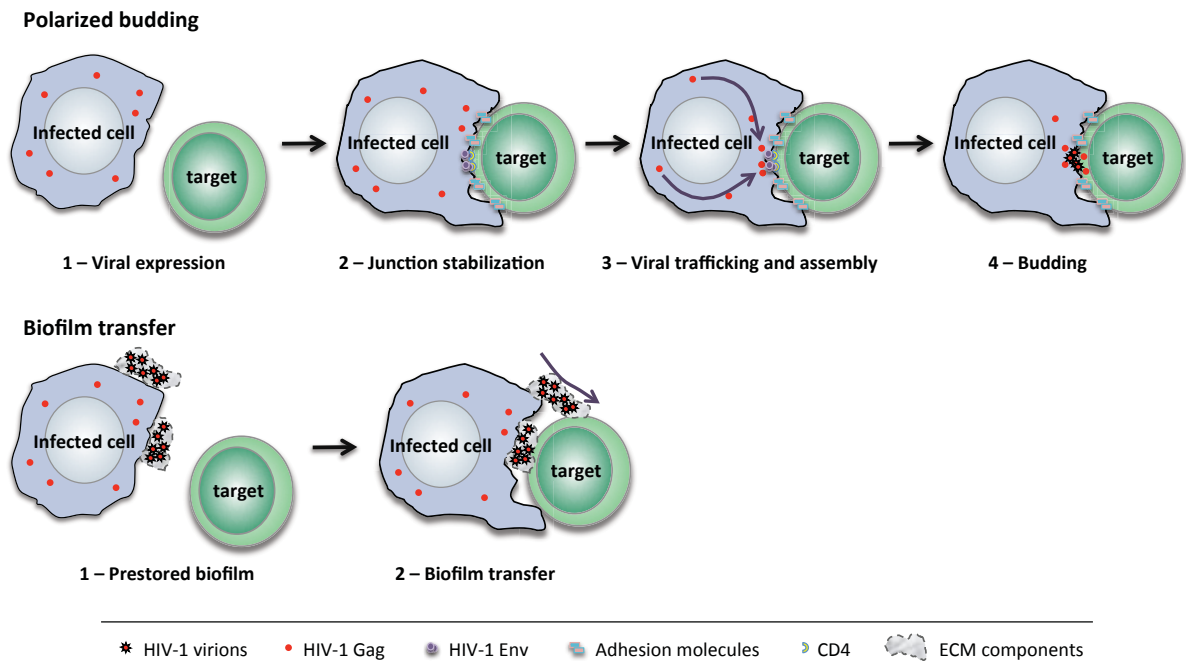
others enables the visualization of aggregates of budded viral particles for wild-type virus as well, either at the interface between target and infected T cells (Jolly et al., 2011; Rudnicka et al., 2009) or away from the junction (Hubner et al., 2009). Nonetheless, their quantitative contribution to the efficiency of cell-associated infectivity was never formally measured. Even, the prevailing paradigm so far rather favored the polarized budding occurring upon formation of intercellular junctions as the main source of HIV-1 T-cell associated infectivity (Jolly et al., 2011). Although some authors already proposed surface transmission to participate in HIV-1 dissemination (Jin et al., 2010), cell-surface retention of viral particles, for instance by host Tetherin, was mainly seen as an obstacle for viral dissemination (Casartelli et al., 2010). Thus, our description of such a major contribution of surface-bound virions to HIV-1 dissemination is to the least surprising. Further, we describe that surface-retention of HIV-1 viral particles and accumulation at the virological synapse is not only mediated by Tetherin but also by ECM components. Another retrovirus, namely MLV, was long thought to be conveyed from infected to target cells only through contact-induced polarized budding (Jin et al., 2009). However, recent work evidences the contribution of surface-bound virions retained by glycosaminoglycans for the efficiency of its transmission. (Li et al., 2014; Sherer et al., 2010). The contribution of a viral biofilm to viral intercellular transmission could thus potentially be extended to other viruses.

What would be the advantage for viral dissemination to occur via surface-bound virions as compared to a polarized budding?

First, pre-storage of viral particles at the cell surface counterbalances potential HIV-1 low viral gene expression that would inevitably limit the pace of synchronized viral assembly and budding. HIV-1 viral burst size is even lower in macrophages (Nasr et al., 2012) and dendritic cells (Steinman et al., 2003) than in T cells. Since those cells are already known to pre-store viral particles in surface-connected compartments (Cavrois et al., 2007; Gaudin et al., 2013), it would be highly relevant to explore the capacity of these two cell-types to produce a viral biofilm.

Second, pre-storage of mature infectious viral particles at the cell surface could represent a kinetic advantage as compared to contact-induced polarized budding at the virological synapse. As it requires junction stabilization (Chen et al., 2007; Jolly et al., 2004; Jolly et al., 2007) and synchronized activation of the cellular machinery regulating viral trafficking, assembly and budding of viral components at the cell surface, contact-induced polarized budding indeed appears as a relatively slow process as compared to surface-

transmission of a viral biofilm (Figure 35). One way to explore this kinetic advantage would thus be to compare the pace of capture by target cells of surface-bound virions versus *de novo* budding viral material.



**Figure 35. Potential advantage(s) of biofilm transfer versus polarized budding.**

*Polarized budding of viral particles is potentially limited in its efficiency by the HIV-1 low viral expression. In addition, it probably requires junction stabilization and synchronized viral trafficking and assembly to ultimately lead to an efficient budding at the contact zone with the target cell. On the contrary, pre-storage of viral particles seems to represent a kinetic advantage over this multi-step process in that it only involves the transfer of a pool of readily preformed infectious viral particles. This pool of virions could have additional properties further enhancing the efficiency of HIV- cell-to-cell transmission.*

Finally, the properties of HIV-1 biofilm itself, in link with the nature of the pre-stored viral particles and the components retaining them at the cell surface, could enhance the efficiency of biofilm-mediated transmission as compared to polarized budding. HIV-1 biofilm indeed appears to provide HIV-1 transmission with three main properties that are further discussed in the following paragraphs, namely: adhesion, protection and infectivity.

## 2. HIV-1 biofilm is a highly adhesive structure

HIV-1 biofilm displays many interesting features potentially implicated in the efficiency of intercellular transmission. Among those properties, adhesiveness represents a keystone for efficient viral dissemination. HIV-1 biofilm indeed represents a highly adhesive structure that has the propensity to adhere, independently of its producing cell, on inert surfaces such as glass coverslips (Figure 28).

Our study unravels the enrichment of HIV-1 biofilm in ECM proteins, and more specifically agrin, collagen, cranin and syndecan 3 (Figure 26). Most of these ECM components are glycosylated proteoglycans that display highly intrinsic adhesive properties and the capacity to specifically interact with a broad range of ligands. In addition, HIV-1 biofilm is enriched in the carbohydrate moiety sLeX (Figure 26) characterized for its participation in intercellular adhesion processes (Liu et al., 2011).

We document the implications of HIV-1 biofilm adhesive properties at many steps of HIV-1 cell-to-cell transmission.

First, we show that ECM components, and more specifically collagen, efficiently retain HIV-1 virions in a cohesive entity at the cell surface, thus conditioning the efficiency of HIV-1 intercellular transmission. Indeed, collagenase digestion of HIV-1 biofilm decreases the amount of surface-associated viral particles and subsequently hampers HIV-1 intercellular transmission (Figure 29). The highly adhesive scaffold formed by ECM components thus ensures the retention and the sustainability of HIV-1 virions at the cell surface. In addition, this ECM-scaffold probably resists shear forces inherent in T cell migration in blood vessels and lymphatics. As migrating infected T cells appear as a vehicle for HIV-1 dissemination (Murooka et al., 2012), the preservation of HIV-1 biofilm cohesiveness at the surface of infected T cells might participate in HIV-1 dissemination throughout the body.

Second, our data suggest that, upon interception with a non-infected cell, HIV-1 biofilm behaves as a glue favoring the establishment of junctions and stabilizing them. Consistent with Jolly *et al.*'s observation of viral clusters at the interface between infected and target T cells (Jolly et al., 2011), we indeed observed HIV-1 biofilm accumulating at the interface between infected and target T cells in  $55 \pm 16$  % of the junctions (Figure 28). Interestingly, although adhesion molecules were shown to accumulate at the VS and to stabilize the junction (Jolly et al., 2004; Jolly et al., 2007), their precise localization was never assessed. Some of these molecules, such as ICAM-1 (Jalaguier et al., 2015), are incorporated in HIV-1 viral particles. Adhesion molecules accumulated at the VS could thus be anchored either in the

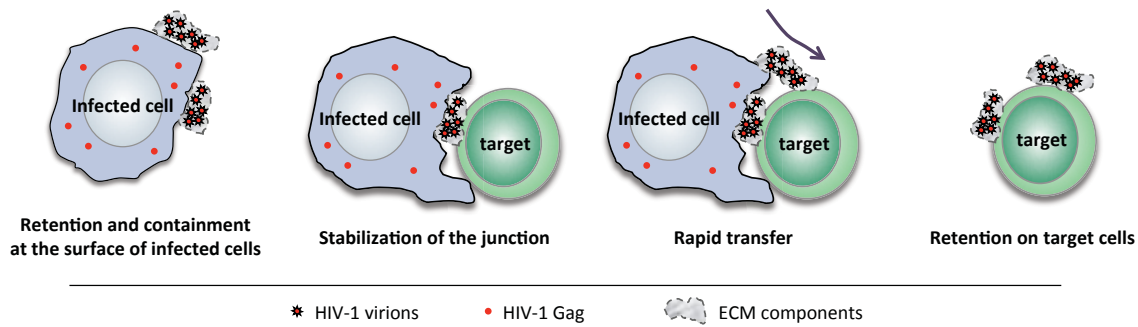
plasma membrane of the infected cell or in the lipid bilayer of HIV-1 viral particles accumulating at the junction. In addition, some incorporated host proteins are known receptors for the ECM and could stabilize the biofilm (see DISCUSSION II.2. Biofilm formation: an intimate interplay between virus, host cell and ECM). Thus, HIV-1 biofilm adhesive properties arise from enriched ECM, from host proteins incorporated in HIV-1 viral particles and from the network they form. Those adhesive properties could participate in the stabilization of intercellular contacts between infected and target T cells and thus favor HIV-1 transmission.

Third, HIV-1 biofilm adhesive properties favor its efficient transfer to target cells as a cohesive entity. We indeed observe that already after 10 min of coculture with infected cells, 5% of target cells have trapped a biofilm (Figure 28). High adhesiveness hence seems to enable a rather rapid and efficient docking of the biofilm to the plasma membrane of the target cell that abolishes the requirement for a long-lasting contact between infected and target cell. Factors favoring the adhesion of the biofilm to the target cell rather than to the donor cell remain to be determined. Nevertheless, biofilm components preserve the cohesion of HIV-1 biofilm during and following its transfer to target T cells.

Fourth, enrichment in ECM components might provide HIV-1 virions with alternative entry pathways that potentially modify viral infectivity. HIV-1 transmission at the virological synapse is thought to proceed *via* endocytosis-mediated entry (Dale et al., 2011). As ECM components enriched in HIV-1 biofilm represent potential HSPG ligands, and since HSPG are well-characterized endocytosis-receptors (Christianson and Belting, 2014), it is likely that local enrichment in ECM participates in an endocytosis-mediated entry of biofilm-associated virions.

Finally, the adhesive properties of HIV-1 biofilm mediate its retention at the surface of the target cell even after dissociation of the junction between donor and target cells. Consistent with the observation that HIV-1 Gag remains clustered at the surface of target cells (Hubner et al., 2009), we report here that HIV-1 biofilm remains associated to the surface of isolated target T cells following its transfer, and that the fraction of target cells carrying a biofilm increases over time (Figure 28). As heparan sulfates are known attachment factors for HIV-1 at the surface of target cells (Roderiquez et al., 1995), adhesive properties of HIV-1 biofilm might thus serve as attachment factors increasing the efficiency of HIV-1 intercellular transmission. Such prolonged association with the plasma membrane of the target cell manages time for subsequent entry or further transmission to another target cell.





**Figure 36. Main consequences of biofilm adhesive properties on HIV-1 cell-to-cell transmission.**

*ECM biofilm components participate in the retention of HIV-1 viral particles at the surface of infected T cells. In addition, HIV-1 biofilm potentially favors the formation and stabilizes the junction between infected and target T cells. Adhesion of HIV-1 biofilm to the target cell enables its rather rapid transfer and its retention at the surface of target cells, enabling further entry or transmission to another target cell.*

Overall, clustering of HIV-1 viral particles in a highly adhesive supramolecular structure might favor its dissemination throughout the body at the surface of producing cells, but also stabilize junctions established with target T cells and favor its capture and retention at the surface of target cells, many features obviously favorable to the efficiency of HIV-1 transmission. Further, this adhesion capacity mediates the trapping of HIV-1 biofilm by non-permissive cells involved in the dissemination of the infection (Figure 31), questioning and ultimately enriching the definition of cell-associated infectivity.

### 3. Cell-associated infectivity: active or passive carriage?

Cell-associated infectivity generally denotes the ability of an infected cell to transfer HIV-1 infection *via* cell-to-cell contact. Here we show that HIV-1 T-cell-associated infectivity mostly resides in the production of a viral biofilm. However, this highly adhesive structure exposed at the surface of infected T cells that can be easily captured by inert surfaces (Figure 28) as well as non-permissive cells (Figure 31) and further transmitted to susceptible cells (Figure 31&Figure 32). Hence, it is legitimate to address whether the definition of cell-associated infectivity mediated by HIV-1 biofilm can be extended to non-permissive cells, non-infected cells or even dead cell. Further, we wonder whether cell-associated infectivity only involves a passive carriage of HIV-1 biofilm or whether it represents a more active process.

In the present work, we show that poorly permissive mature dendritic cells (DCs) capture HIV-1 biofilm and enhance its infectivity to non-infected target T cells (Figure 31). However, the *trans*-infection effect we observe could potentially be mediated by enhanced clustering of infected and target T cells mediated by mDCs, thus enhancing the probability and efficiency of biofilm transfer directly from one T cell to another. As we observe that mDCs are able to capture HIV-1 biofilm, and since we never observed such T-T contacts at the surface of mDCs, we are confident that DCs are able to transfer the biofilm they captured to target T cells. A practical way to ensure our intimate conviction would be to remove biofilm-producing T cells from our experimental set-up following biofilm capture by DCs.

Provided that non-infected DCs really display the ability to vehicle HIV-1 infectivity in a biofilm-mediated manner, it remains to be determined whether this carriage is a genuine passive carriage. Indeed, while collagenase digestion of HIV-1 biofilm reduces the efficiency of T-T transfer by up to 60% (Figure 29), similar digestion hampered the efficiency of *trans*-infection by only 30% (Figure 31). It is thus possible that mDCs compensate the lack of collagenase by alternative means that would counterbalance collagenase-disruption of HIV-1 biofilm integrity (Figure 30), through viral particles clustering and/or enrichment of the biofilm with additional ECM components. mDCs are indeed known to cluster viral particles in membrane invaginations prior to their transfer to target T cells, and this clustering could be mediated by ECM components. A valuable approach to evaluate the contribution of mDCS ECM to HIV-1 biofilm could have been to stain HIV-1 captured biofilm at the surface of DCs with antibodies targeting DC-specific ECM components such as syndecan 3 (de Witte et al., 2007).

Other non-permissive cells than mDCs represent good carriers for HIV-1 virions such as spermatozoa (Ceballos et al., 2009), epithelial cells (Bomsel, 1997; Ganor et al., 2010) or follicular dendritic cells (Banki et al., 2005). As spermatozoa capture HIV-1 through syndecans (Ceballos et al., 2009), epithelial cells transcytose HIV-1 virions in an agrin-mediated manner (Alfsen et al., 2005) and follicular dendritic cells produce large amounts of ECM proteins (Mabbott et al., 2011), those non-permissive cells represent potential carriers for HIV-1 biofilm that would require further exploration.

When carried at the surface of mDCs, HIV-1 cell-associated infectivity mediated by HIV-1 biofilm hence does not necessarily require an infection. Further, our data suggest that non-infected T cells as well can represent carriers for cell-associated infectivity. We show that both in the context of T-T transmission and in DC-mediated *trans*-infection, infectivity of non-infected cells complemented with free virus surpasses that of free virus alone (Figure 32). As non-infected cells are unlikely to complete a round of viral amplification within the time-frame of the experiment, this increase in free virus infectivity observed upon addition of non-infected T cells is likely due to the carriage and possible clustering of viral particles by non-infected T cells. Incubation of non-infected CEM T cells with labeled viruses would have unraveled their potential ability to capture, coalesce and expose viral particles in a pseudo-biofilm at their cell-surface. Non-infected T cells hence represent potential active carriers of HIV-1 biofilm-mediated infectivity.

As dead infected T cells can still transmit infection to macrophages (Baxter et al., 2014), it would be interesting to explore whether dead biofilm-carrying T cells are still able to transfer an infectious biofilm. In an attempt to challenge this hypothesis, we tried to kill infected biofilm-producing cells through serum starvation but never succeeded.

Overall, is HIV-1 biofilm transfer a passive or active process? HIV-1 biofilm transfer could indeed represent a passive process whereby cell-surface expression of adhesion molecules by the target cell would be sufficient to capture and retain the biofilm and surpass the retention forces deployed by the donor cell. Alternatively, biofilm transfer could constitute an active process requiring the cleavage of molecules mediating the adhesion and retention of HIV-1 biofilm at the surface of the donor cell. As HIV-1 infection upregulates the expression of matrix metalloproteases (MMP), especially MMP-2 and -9 (Louboutin et al., 2011) that are able to cleave ECM components, otherwise enriched in HIV-1 biofilm, it would be of high interest to explore the role played by MMPs in the intercellular transfer of HIV-1 biofilm.

In conclusion, most of HIV-1 T-cell-associated infectivity resides in a highly adhesive viral biofilm exposed at the cell surface. This biofilm can subsequently undergo a carriage by

either the producing cell or a bystander cell that most certainly participates in the infectivity of the biofilm it carries, either by adding some ECM components or by favoring its active transfer to target cells and anyway, by enlarging and diversifying the range of potential target cells explored by this biofilm. However, contribution of the carrier cell is comparable to the potency of biofilm effect *per se* (Figure 31C) and some features of HIV-1 biofilm most certainly condition the intrinsic infectivity of the biofilm.

## II. ECM biofilm components enhance HIV-1 infectivity

### 1. Biofilm ECM components harbor viral particles in a structured protective microenvironment

As collagenase-induced decrease in HIV-1 transmission correlates to a reduction in cell-associated viral material (Figure 29), ECM components contribute to HIV-1 infectivity through the retention of HIV-1 virions in a viral biofilm exposed at the surface of T cells. However, this viral biofilm provides HIV-1 transmission with additional properties independent of its retention capacities. For instance, Heparin competition decreases the efficiency of HIV-1 transmission without affecting the amount of cell-associated material (Figure 29), suggesting a retention-independent contribution of heparan sulfates to HIV-1 transmission. As Heparin is reported to compete with HIV-1 attachment as well (Guibinga et al., 2002), we extensively washed the cells in order to remove heparin, and checked the efficiency of this removal by controlling the absence of inhibitory effect of medium used for the last wash. The contribution of heparan sulfates should be further characterized using siRNA targeting heparan sulfates, for instance agrin, a strategy that we so far did not get to efficiently work in our T cell systems. If reduced infectivity is truly not linked to reduced retention of virions, heparan sulfates might participate in biofilm infectivity either by their intrinsic nature or through the specific organization they confer to HIV-1 biofilm, especially upon transfer to target cells. More generally, through the microenvironment they build in the biofilm, ECM components might provide HIV-1 virions with additional properties such as a regulated architecture, a protective shield, alternative entry pathways as well as soluble potentiating factors.

First, ECM participation in biofilm infectivity could be partially linked to its architecting role within HIV-1 biofilm. Scanning Electron Micrographs of HIV-1 biofilm adsorbed on glass coverslips indeed display threads that connect virions with one another (Figure 28). Further, collagenase digestion seems to unpack HIV-1 biofilm (Figure 30), potentially participating in decreased HIV-1 intercellular transmission. Indeed, while collagenase digestion triggers a 60% decrease in infectivity, it impairs HIV-1 retention at the cells-surface only by 25% (Figure 29), indicating the participation of additional factors, architecture of the biofilm representing a valuable candidate. In an attempt to quantitatively analyze the morphology of HIV-1 biofilm in the absence of some ECM components, we

initiated the development of a script enabling the automated quantification of HIV-1 biofilm morphologic parameters on confocal images using Acapella software. Should the enhancing effect of heparan sulfates or collagen rely on the specific architecture they provide to HIV-1 biofilm, modifications in biofilm architecture could be monitored by performing super-resolution microscopy like SIM on the formation and transfer of a biofilm devoid of those ECM components.

Second, this organized microenvironment might represent a protective shield preserving viral particles infectivity over time and towards neutralizing antibodies. Regarding the preservation of HIV-1 infectivity over time, our preliminary data indicate a constantly higher infectivity over time for biofilm-associated viral particles as compared to equal amounts of cell-free viral particles. However, this experiment should be reproduced for confirmation due to high inter-experiment variations in the absolute infectivity of biofilm and free viral particles. As HIV-1 plasmatic virions have a dramatically short life span of 0.3 days (Perelson et al., 1996), the protective microenvironment formed by ECM components would represent an undeniable advantage for HIV-1 infectivity. In addition, ECM components are mainly highly glycosylated proteins. Their enrichment in HIV-1 biofilm constitutes a second-glycan shield that contributes to the protective effect of HIV-1 Env glycosylated canopy. As glycosylations, especially when carried by ECM proteins encoded by the self-genome, are immunologically silent and represent hindrances to the accessibility by neutralizing antibodies (NAbs), ECM components of HIV-1 biofilm may shelter HIV-1 viral particles from NAbs recognition. So far, we show that the infectivity of HIV-1 biofilm is preserved even in the presence of 2G12 concentrations otherwise impairing cell-free infectivity (Figure 34). Isolated from an Elite Controller, NAb 2G12 binds a cluster of two to four high-mannose ( $\text{Man}_8\text{GlcNAc}_2$  and/or  $\text{Man}_9\text{GlcNAc}_2$ ) glycans (Mouquet, 2014) and could potentially bind similar glycans carried by ECM proteins. Extending our study to bNAbs would be of high clinical interest. Further, we show that, although they access viral particles as assessed by RT-SEM, gold nanoparticles coupled to the Fc fragment of anti-Env antibodies seem to be inaccessible to SEM imaging in cryo conditions (see Appendix 4). These results indicate that even if anti-Env antibodies (and by extension NAbs) make their way to their epitope within HIV-1 biofilm, it is likely that their Fc fragment would be too short to point out of the biofilm for subsequent recognition by membrane-bound Fc-receptors. NAbs would thus be inefficient at inducing their potent ADCC and ADCVI effects. ECM enrichment in HIV-1 biofilm might thus represent a second level of protection from the immune system.

Finally, besides its structuring properties, biofilm ECM meshwork traps a broad range of soluble factors that might potentiate viral particle infectivity. Our TEM experiments on HIV-1 biofilm enable the visualization of some particles devoid of dense-core (Figure 23) that might represent exosomes. Loaded with a broad range of micro-RNAs and proteins, exosomes constitute a potent mean of intercellular communication modulating the expression profile of recipient cells (Villarroya-Beltri et al., 2013). Also known to modulate the expression of a broad range of host protein, Tat binds HSPG and can enter non-infected cells by HSPG-mediated endocytosis (Tyagi et al., 2001). Similarly, chemokines are efficiently captured by heparan sulfates (Sadir et al., 2004) and could exert their effect within HIV-1 biofilm, potentially attracting target T cells towards the biofilm. It would be of high interest to test by immunofluorescence the presence of chemokines, Tat and exosomes within HIV-1 biofilm. If they are enriched in HIV-1 biofilm, the meshwork of ECM components within HIV-1 biofilm would constitute a net trapping and preserving factors favoring the attraction of target cells and potentially modulating their pattern of expressed proteins to potentiate the efficiency of *de novo* infection.

Overall, the meshwork of ECM components enriched in HIV-1 biofilm builds a structured and protective microenvironment, probably enriched in potentiating soluble factors that might contribute to the enhancement of HIV-1 infectivity and dissemination.

## **2. Biofilm formation: an intimate interplay between virus, host cell and ECM**

Although ECM enrichment in HIV-1 biofilm confers HIV-1 with fundamental properties, HIV-1 biofilm properties do not solely arise from ECM components it contains but also from the intimate cross-talk between the virus, its host cell and ECM components.

Ability to produce a biofilm is thus likely resulting from the co-evolution and the intimate interplay between HIV-1 and its host cells. As ECM patterns expressed as well as host proteins incorporated within HIV-1 virions vary depending on the cell type considered, we decided to focus our study exclusively on HIV-1 biofilm produced by T cells. Interestingly, ability to form a biofilm was a constant and shared feature of all T cell systems as well as all HIV-1 strains we studied (Figure 22 & Table 4), both in the context of acute and chronic infection. Biofilm formation thus represents as a robust feature independent of the establishment of chronicity, as could have been inferred following the work on HTLV-1 biofilm (Pais-Correia et al., 2010).

ECM proteins (Sung et al., 2011) and viral components (Caillet et al., 2011; Murray et al., 2005) have been independently shown to be exported through similar pathways, involving the endosome recycling machinery. As we find uropod marker CD44 enriched in HIV-1 biofilm (Figure 26), HIV-1 biofilm production most probably occurs at the uropod where viral particles are reported to bud in T cells (Llewellyn et al., 2010). Interestingly, for parasites such as *Entamoeba histolytica*, the uropod constitutes a cellular structure where the cell extrudes components otherwise harmful (Girard-Misguich et al., 2008). As HIV-1 infection is highly cytopathic (Doitsh et al., 2014), clearing the cytoplasm from viral components as they get produced might be beneficial to the survival of the infected cell, especially in a context of chronic infection. It is thus possible that coalescence of HIV-1 virions with ECM components represents a fortuitous consequence of their co-secretion at the uropod of T cells. This hypothesis could be verified through live imaging of biofilm formation in cell lines expressing fluorescently-labeled viral and ECM components and silenced for major actors of the endosomal recycling pathway. However, as only specific components are modulated by HIV-1 infection and accumulated within HIV-1 biofilm, it is likely that this interplay with the ECM represents a tightly regulated process, possibly involving HIV-1 accessory proteins. A post-doctoral fellow in our team, Marina Caillet, is currently challenging the contribution of HIV-1 accessory proteins in the regulation of biofilm formation.

Another aspect of the intimate cross talk between HIV-1 and its host cell involved in biofilm formation resides in the adhesion processes driving HIV-1 biofilm cohesion. Although ECM proteins are able to interact with one another to form an organized meshwork, the way HIV-1 viral particles are retained within HIV-1 biofilm is unlikely to be the sole of their passive trapping preventing their release. Retention of viral particles within the ECM meshwork may be mediated by interactions of ECM components with both HIV-1 Env glycoprotein and resident host proteins incorporated within HIV-1 viral particles. HIV-1 gp41<sup>TM</sup> and human agrin display the ability to interact with one another (Alfsen et al., 2005), indicating an adaptation of the virus to its host cell to accommodate interactions potentially stabilizing HIV-1 biofilm. The requirement for Env incorporation in biofilm formation is currently challenged. In addition, HIV-1 viral particles incorporate a broad range of host proteins upon assembly and budding and among them some are known receptors of the ECM such as ICAM-1 (Languino et al., 1995) and CD44 (Ishii et al., 1993; Jalkanen and Jalkanen, 1992; Lesley and Hyman, 1992). Those ECM binding partners are also likely to participate in the stabilization of HIV-1 biofilm. Stabilizing role of ICAM-1 and CD44 in biofilm formation could be challenged through competition with neutralizing antibodies.



Overall, a viral biofilm is not only the sum of its components, ie viral particles and ECM proteins. A viral biofilm rather embodies the complex interplay between viral infection and infected cells, involving the modulation of ECM production pathways and interactions between viral particles and ECM components, ultimately fine-tuning viral biofilm composition and architecture and thus conditioning its infectivity. Nevertheless, it remains to be determined whether ECM modulation and subsequent enrichment in HIV-1 biofilm should be regarded as a virus-driven process favoring HIV-1 cell-to-cell spread or as an encysting process orchestrated by the cell to prevent the release of viral particles.

### **3. ECM: a restriction factor or a transmission factor?**

Current vision of the participation of ECM components in the physiopathology of HIV-1 infection mostly focuses on the fibrotic damage induced by collagen up-regulation (Smith et al., 2010) and subsequent deposition in the lymphatic tissues, that apparently limits CD4+ T cell repopulation (Estes, 2009) and impairs the immune function of secondary lymphoid organs (Beghini et al., 2015). As migratory T cells vehicle HIV-1 infection throughout the body (Murooka et al., 2012), prevention of lymphocyte migration by fibrosis thus appears as a negative factor for the spread of the infection. However, matrix metalloproteases (MMPs) are largely upregulated by HIV-1 infection, correlating to accelerated disease progression (Mastroianni and Liuzzi, 2007). Selective MMP-mediated ECM processing could release bioactive fragments that would enhance lymphocyte migration (Korpos et al., 2010), thus counterbalancing the apparent reduced cellular motility induced by HIV-1 accessory protein Nef (Nobile et al., 2010) and ultimately favoring viral dissemination by migratory infected T cells. Overall, ECM contribution to viral dissemination at the scale of the organism is a rather complex mechanism involving both positive and negative impacts.

Regarding their contribution to HIV-1 cell-to-cell spread, ECM components have always been considered from the carrier or target cell point of view. Many reports hence characterize some ECM components as attachment factors favoring HIV-1 capture and subsequent carriage by non-permissive cells (de Witte et al., 2007) or entry into target cells (Ohshiro et al., 1996). Their contribution from the infected cell point of view remained to be characterized. Our data demonstrate the crucial contribution of heparan sulfates and collagen enriched in HIV-1 biofilm to the efficiency of HIV-1 transmission (Figure 29). ECM

components enriched in HIV-1 biofilm hence represent factors favoring HIV-1 intercellular transmission.

Our study indicates that ECM components are accumulated in HIV-1 biofilm in a regulated and specific manner. The present work indeed evidences that specific ECM components, such as collagen, agrin, syndecan-4, cranin and sLeX are specifically enriched in HIV-1 biofilm (Figure 26). In addition, some ECM components accumulated in HIV-1 biofilm are upregulated upon HIV-1 infection, such as syndecan-4 (see Appendix 3) and collagen (Figure 24), while other such as agrin, have a globally unmodified expression pattern as compared to non-infected cells (Figure 24). Further, not all ECM components expressed by HIV-1 infected T cells are accumulated in the biofilm, as galectin3 seems to accumulate in other regions of the plasma membrane (Figure 26). Enrichment of HIV-1 biofilm in specific ECM components thus represents a tightly regulated process. Interestingly, HTLV-1 biofilm was shown to be enriched in agrin and collagen as well, but also in galectin-3 (Pais-Correia et al., 2010). In addition to a morphology that appeared more packed than that of HIV-1 biofilm, similarities and discrepancies between HIV-1 and HTLV-1 biofilm compositions indicate convergences but also differences in the mechanisms regulating biofilm formation for those two retroviruses.

In addition, we show that host protein Tetherin also contributes to the retention of WT HIV-1 virions at the cell surface (Figure 27). As viral accessory protein Vpu is inducing Tetherin degradation (Neil et al., 2008), detection of Tetherin in CD4<sup>+</sup> T cells infected with WT viruses was unexpected. While cell-to-cell transmission appears to circumvent Tetherin retention of HIV-1 virions at the cell surface (Zhong et al., 2013), an excess of Tetherin inhibits HIV-1 cell-to-cell transfer (Casartelli et al., 2010). It is likely that, similarly, the nature and the relative quantity of ECM components retaining HIV-1 virions at the cell surface condition the efficiency of viral transmission. In the absence of Tetherin, we show that the fraction of cells carrying a biofilm is reduced but that more biofilms are enriched in collagen (Figure 27). Tetherin thus contributes to surface retention of about 14% of HIV-1 biofilms while the remaining fraction might be retained by other components such as collagen. This observation seems to indicate that equilibrium between biofilm components such as collagen and Tetherin is required for the optimal retention of HIV-1 virions at the cell surface and their subsequent transmission by cell contact. Further studies combining functional assays and super-resolution microscopy are required to delineate the precise contribution of each biofilm component to viral particle retention and transmission.

Overall, this work broadens the current vision of ECM contribution to HIV-1 dissemination and unravels its crucial contribution to HIV-1 cell-to-cell spread through its enrichment in HIV-1 biofilm. Biofilm enrichment in ECM proteins constitutes a tightly regulated process in which the relative quality and quantity of biofilm components balances viral particle retention and HIV-1 intercellular spread.

### III. HIV-1 biofilm formation potentiates viral particles infectivity

HIV-1 transmission by intercellular contacts is far more potent than its dissemination through free-viral particles (Chen et al., 2007; Dimitrov et al., 1993). However, mechanisms underlying this increased infectivity are not fully explored yet. We show here that HIV-1 cell-associated infectivity mostly relies on surface-bound viral particles exposed at the surface of T cells in a viral biofilm (Figure 20 & Figure 25). In parallel, we report that biofilm-associated virions are more infectious than equal amounts of cell-free virions, both in direct T-T transfer and in DC-mediated *trans*-infection (Figure 32). We thus unveil a new cause for the increased efficiency of HIV-1 transmission by cell contacts. Further, we measure HIV-1 biofilm contribution to reported decrease sensitivity to ArT of HIV-1 cell-to-cell transfer (Figure 33). Since isolated biofilms retain their increased infectivity even in the absence of the producing cell (Figure 32), this feature appears as an intrinsic property of HIV-1 biofilm. As discussed above, spread of HIV-1 virions most certainly benefits from their peculiar organization with specific ECM components within the biofilm. Nonetheless, complementary factors could account for the potentiation of their infectivity within HIV-1 biofilm. We will discuss below the potential different nature of biofilm-associated virions as compared to their cell free counterparts and the potential benefit of viral particle clustering within a biofilm.

#### 1. Nature of the viral particles might be different

About one fifth of the viral progeny of a cell culture is retained within HIV-1 biofilm while the vast majority is released as cell-free viral particles in the supernatant (Figure 20). What drives the accumulation of HIV-1 virions in a viral biofilm instead of their release as “planktonic” virions? Anyway, are cell-free viral particles really produced as isolated “planktonic” virions? Some of our SEM observations indicate that clusters of 2 to 10 viral particles associated to agrin can be found in cell-culture supernatant of chronically infected T cells. In addition, the reported association of HIV-1 virions to ECM proteins (described in Appendix 2) could constitute a hallmark of their journey through the biofilm. Shear stress, transient intercellular interactions or even active regulation of the size of the biofilm could induce the release of pieces of biofilm composed of single planktonic virions along with small clusters of viral particles.

Alternatively, is accumulation within the biofilm a matter of kinetic and synchronization of viral particle budding with ECM production and trafficking? Are cell-free and biofilm-associated viral particles comparable, for instance in their intrinsic infectivity and ability to stably interact with ECM proteins? Several intrinsic properties condition the infectivity of HIV-1 viral particles, mainly a successful maturation and reorganization of the fullerene core, along with a sufficient number of functional fusogenic-competent Env glycoproteins (Joyner et al., 2011; Layne et al., 1992; Murakami et al., 2004). Incorporation of host proteins within viral lipid bilayer might as well participate in virion infectivity. These aspects could simultaneously favor association to HIV-1 biofilm.

Our confocal experiments reveal that HIV-1 biofilm is composed of mature MAp17+ viral material (Figure 22) and TEM and FIB/SEM unravel the presence of dense-core mature viral particles within HIV-1 biofilm (Figure 23 & Figure 25). Although requiring a proper quantification, the maturation rate of virions within HIV-1 biofilm appears rather high as compared to the reported 40% maturation for cell-free viral particles collected from culture supernatants (Ohishi et al., 2011). Careful scrutiny of HIV-1 extracellular pools observed by others enables to determine a maturation rate of  $61 \pm 33$  % for surface-bound virions (Ladinsky et al., 2014), indicating a favored maturation as compared to virions released as cell-free particles. Apparent high maturation rate within HIV-1 biofilm has two possible explanations. Biofilm microenvironment might harbor biochemical and biophysical conditions favorable to PR activation and processing. Alternatively, mature viral particles could be actively sorted in within HIV-1 biofilm following PR activation, for example through maturation-induced conformational changes in HIV-1 Env (Murakami et al., 2004), that would alter its interaction capacities with ECM biofilm components. Analysis of biofilm formation by a maturation-deficient virus, for example mutated in PR catalytic site or in the presence of a protease inhibitor, would enable to decipher whether maturation is a prerequisite for incorporation of viral particles within HIV-1 biofilm. As protease processing increases HIV-1 Env fusogenic capacities (Murakami et al., 2004), apparent increased maturation rate within HIV-1 biofilm probably potentiates viral particle infectivity.

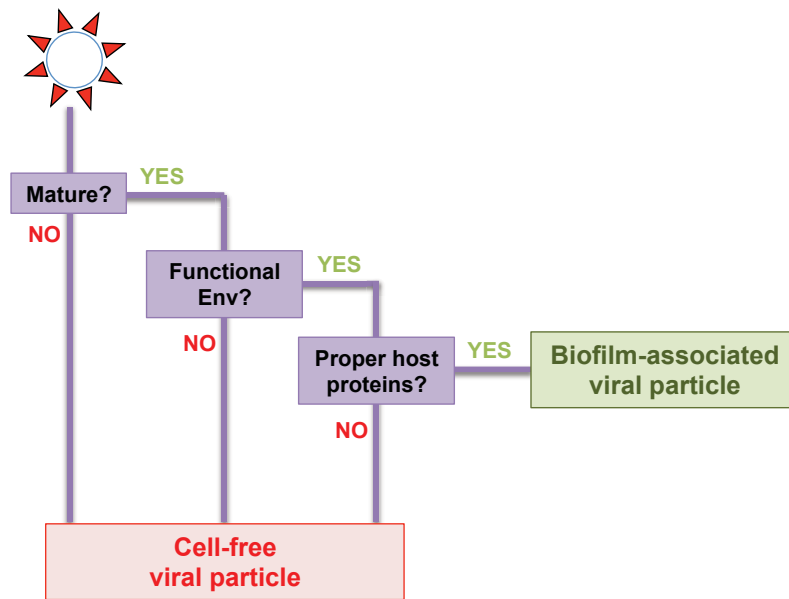
Further, confocal and SEM imaging demonstrate that HIV-1 virions accumulated within HIV-1 biofilm have successfully incorporated Env glycoprotein. As HIV-1 gp120<sup>SU</sup> from certain HIV-1 strains is prone to dissociate from gp41<sup>TM</sup> (Layne et al., 1992; Schneider et al., 1986), packing of HIV-1 viral particles within the dense biofilm microenvironment could

prevent Env shedding. Although preliminary western-blot experiments do not seem to indicate a specific enrichment in gp120<sup>SU</sup> for virions within HIV-1 biofilm, more effort should be put the comparison of Env numbers, nature, conformation and functionality between cell-free and biofilm-associated virions, if Env is found required for biofilm formation. Again, divergences could result from the properties of the biofilm microenvironment that would preserve or enhance Env functionality. For instance, reported binding of agrin to HIV-1 Env (Alfsen et al., 2005) could induce conformational changes in HIV-1 Env favorable to viral particle infectivity or stabilization. Alternatively, upon assembly and budding, viral particles might incorporate Env proteins with divergent conformations, some of which predisposing to interactions with biofilm components and fortuitously being more fusion-competent.

Finally, we find transmembrane host protein CD44 accumulated in HIV-1 biofilm. Receptor for numerous ECM proteins such as hyaluronan (Lesley and Hyman, 1992), fibronectin, laminin and collagen (Jalkanen and Jalkanen, 1992), CD44 is reported to incorporate within HIV-1 virions (Bastiani et al., 1997; Bregnard et al., 2013; Chertova et al., 2006; Stephenson et al., 2010) and to keep its functionality within viral lipid bilayer (Guo and Hildreth, 1995). Biofilm-associated virions presumably harbor several other functional host proteins, possibly belonging to a different pool than the one incorporated by their cell-free counterparts. Some of these host proteins, along with favoring interactions with biofilm ECM components, could favor virions attachment to target cells and thus increase their infectivity. For instance, it would be of high interest to compare the relative enrichment of cell-free VS biofilm-associated virions in ICAM-1, able to bind both fibrinogen within the ECM (Languino et al., 1995) and LFA1 at the surface of the target cell.

Either through increased maturation rate, through preserved Env functionality or by a different pool of incorporated host proteins, biofilm-associated virions might be different in nature from their cell-free counterparts, thus explaining both their accumulation in HIV-1 biofilm and their increased infectivity, as shown in Figure 37.

Last, it is worth to note that we have so far evaluated the relative effect of ArT 3TC and Raltegravir (Figure 33) and NAb 2G12 (Figure 34) on equal amounts of cell-free VS biofilm-associated viral particles. If the nature of those viral particles is different, it would be of high interest to compare the effect of ArT and NAb at equal infectivity. Different nature in viral particles will most certainly lead to a differential response of cell-free and biofilm-associated viral transmission in the presence of those inhibitors.



**Figure 37. Decision-making scheme for viral particle association to HIV-1 biofilm.**

*Accumulation of HIV-1 viral particles within HIV-1 biofilm might be conditioned by successful maturation, incorporation of a sufficient amount of Env as well as incorporation of specific functional host proteins. The proposed scheme does not infer a preponderance of one of those aspects above the others.*

## 2. HIV-1 biofilm: a viral community?

Efficiency of HIV-1 transmission at the virological synapse correlates with the amount of viral material accumulated at the interface between infected and target cell (Zhong et al., 2013). In parallel, HIV-1 ratio of detectable infectious units to virus particles is on the order of 1:1,000 to 1:100,000 for cell-free viral particles (Munch et al., 2007). Increased efficiency of HIV-1 cell-to-cell transmission could thus rely on the local increase in infectious titers mediated by the aggregation of viral particles within HIV-1 biofilm.

Here we show that HIV-1 biofilm is composed of clusters of dozens of viral particles (Figure 23, Figure 28 & Figure 32). Biofilm-mediated aggregation thus locally increases HIV-1 infectious titers at the surface of infected T cells, potentially optimizing the probability of productive infection for one encountered target T cell as compared to cell-free virions. Regarding the link between the size of viral clusters and the efficiency of transmission, our present study does not allow to discriminate the relative contribution of small versus large aggregates of surface-bound virions. However, mechanical washes of biofilm-producing cells seem to reduce the size of HIV-1 membrane-associated clusters. In an attempt to correlate the

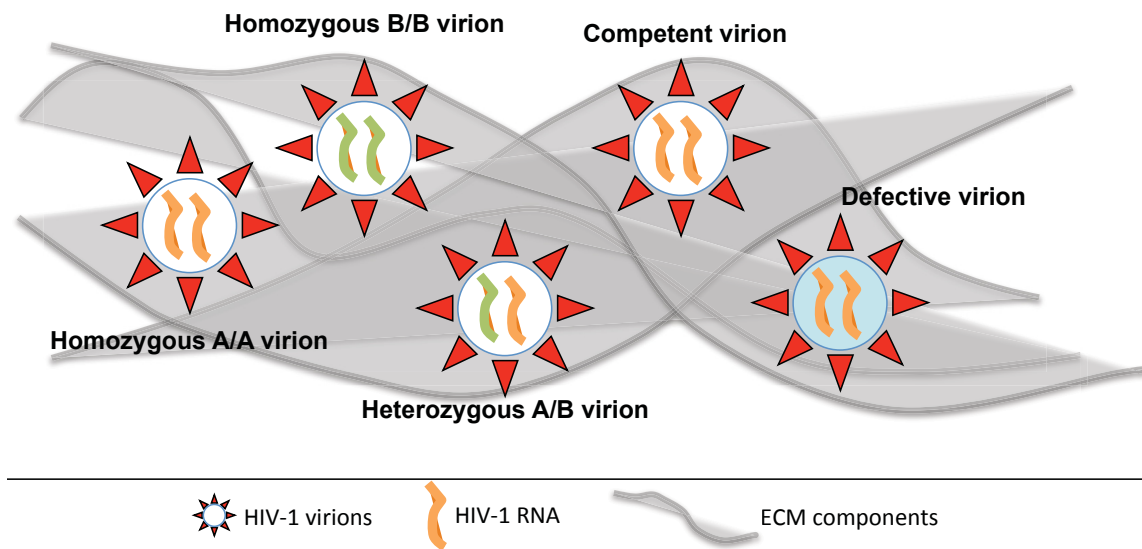
efficiency of HIV-1 cell-to-cell transfer to the size of surface-bound viral aggregates, we initiated the development of a script enabling the automated quantification of the size of HIV-1 biofilm on confocal images using the Acapella software. Preliminary data confirm our visual observations and indicate that mechanical washes of HIV-1 biofilm lead to a 20% decrease in the size of extracellular aggregates, suggesting a crucial role for large clusters in the efficiency of HIV-1 cell-to-cell transfer.

Dendritic cells are already known to cluster viral particles prior to their transmission to target cells (McDonald et al., 2003). Here we show that they also have the ability to capture preformed viral aggregates composing HIV-1 biofilm and to potentiate their transmission to target T cells (Figure 31). As both T cells (Murooka et al., 2012) and DCs can potentially vehicle HIV-1 infection throughout the body, HIV-1 biofilm could mediate the transmission of high infectious titers at several steps of HIV-1 dissemination, involving either direct T-T transmission or DC-mediated *trans*-infection events. As we observe that HIV-1 biofilm contributes to a reduced sensitivity to ArT (Figure 33), biofilm-mediated high infectious titers might favor multiple entry events, proposed to participate in the escape from ArT (Sigal et al., 2011). Multiple entries mediated by HIV-1 biofilm would ultimately lead to the multiple integration events yielded by HIV-1 cell-to-cell transfer (Del Portillo et al., 2011; Duncan et al., 2013; Russell et al., 2013). As biofilm-mediated transmission persists even in the presence of ArT (Figure 33), deeper analysis of the TransBioHIV cohort we started, especially of the 3-month follow-up samples (see Appendix 3), will provide further insights into whether HIV-1 biofilm persists *in vivo* in ArT-treated patients.

In addition to generating a local high infectious titer, clustering of viral particles within HIV-1 biofilm could potentiate infectivity through clustering of different HIV-1 variants. HIV-1 infected cells can indeed carry more than one integrated copy of HIV-1 provirus (Jung et al., 2002), sometimes even belonging to different HIV-1 clades. Provided a simultaneous expression of those proviruses, HIV-1 biofilm could be composed of a mixture of homozygous or even heterozygous diploid virions arising from different integration events, as represented in Figure 38. Ability of HIV-1 biofilm to harbor different genetic variants could be challenged through observation of biofilm produced by T cells co-infected with fluorescently tagged viruses in two different colors. Subsequent infections mediated by such a mixed biofilm could then lead to simultaneous entry of different viral RNA genome, favoring recombination events and thus contributing to the emergence of Circulating Recombinant Forms in the next infection round. These CRF could contribute to the appearance or the



expansion of resistances to ArT and NAb within viral progeny. In addition, defective variants could even benefit from the coinfection with infectious viral particles (Figure 38), that would provide them with viral factors they lack for their proper replication and thus rescue their infectivity potential. Just like bacterial biofilms, HIV-1 biofilm hence represents a microbial community potentially favoring viral dissemination.



**Figure 38. HIV-1 biofilm as a potential viral community.**

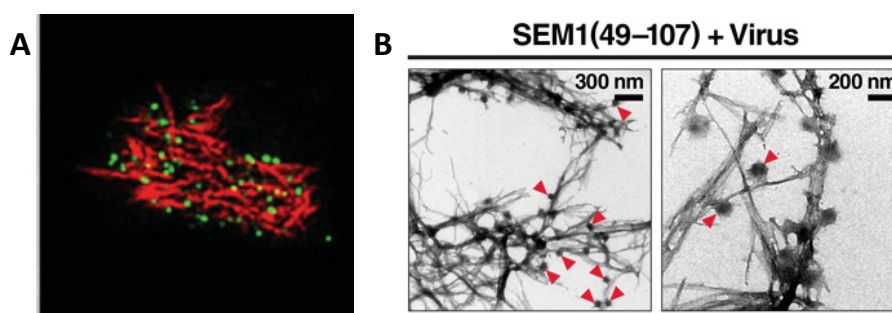
*HIV-1 biofilm could be composed of a mixture of competent and defective virions that could benefit from simultaneous entry events. In addition, if produced by an infected T cell harboring two different integrated proviruses A and B, HIV-1 biofilm could potentially contain homozygous virions from both strains A and B, as well as heterozygous virions A/B.*

### 3. Viral particle aggregation, a general mechanism of dissemination?

Along HIV-1 dissemination throughout the body, some key steps seem to require a clustering increasing the multiplicity of entry. Scientific literature is thus becoming an increasingly prolific source of examples for molecules reported to increase HIV-1 infectivity through viral particle aggregation.

For instance, human defensins HD5 and HD6 enhance HIV attachment by concentrating virus particles on the target cells, thus locally increasing infectious titers along with attachment (Rapista et al., 2011). Although semen can be seen as a deleterious factor for viral infectivity (Sabatte et al., 2007), some semen components were recently characterized for their ability to enhance HIV-1 infection through aggregation. Amyloid fibrils formed by semen-derived enhancer of viral infection (SEVI) were indeed proved to aggregate HIV-1 virions, as shown in Figure 39A, and to subsequently enhance HIV-1 infection of PBMCs by

up to 400,000-fold as compared to virus alone (Munch et al., 2007). Similarly, fibril-forming semenogelins (SEMs) from semen aggregated HIV-1 virions, as depicted in Figure 39B, and enhanced fusion of HIV-1 virions to primary CD4+ T cells (Roan et al., 2011). Interestingly, fibronectin synergistically increased the infectivity enhancement activity of semen amyloids (Roan et al., 2014). As fibronectin is an ECM components and since the morphology of those aggregates is strikingly reminiscent of the morphology we observe for HIV-1 biofilms adsorbed on glass coverslips (Figure 32F), we wonder whether HIV-1 virions are carried in a viral biofilm within the semen. Efficiency of lectin-based microbicides (Huskens and Schols, 2012), known to bind glycosylations also harbored by ECM components, further support the importance to test the presence of HIV-1 biofilm in semen from HIV-1 patients.

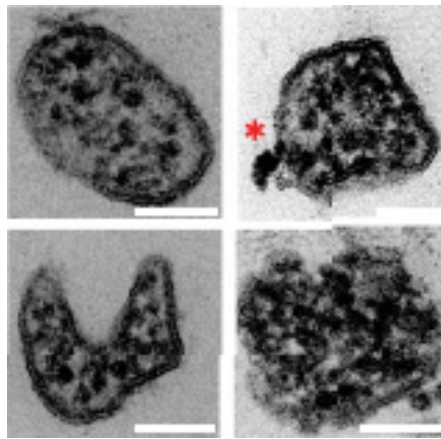


**Figure 39. Imaging of HIV-1 viral particle aggregation in semen.**

*A. Fluorescence imaging of semen amyloids SEVI (red) clustering HIV-1 viral particles (green) (left panel from (Usmani et al., 2014) and right panel from (Allen et al., 2015)). B. Transmission electron microscopy of SEM amyloid fibrils clustering HIV-1 viral particles (red arrowheads) (from (Roan et al., 2011)).*

Aggregation of HIV-1 virions is also used in laboratory practice to enhance infection efficiency. Hence, Poly-L-lysine (PLL) potentiating effect on infection efficiency is partially due to its ability to aggregate virions, with a 15- to 20- fold increase in effective particle diameter (Davis et al., 2004) enhancing adsorption *via* sedimentation (Davis et al., 2004). Similarly, a genetically engineered assembled peptide termed CD4bs-M increases HIV-1 infection by up to 8-fold. In addition to possible conformational changes within Env, facilitation of infection was mainly attributed to the ability of CD4bs-M to aggregate viral particles (Gross et al., 2015).

Recent studies enrich our view that viral particle aggregation constitutes a key driver of infectivity, for viruses other than retroviruses as well. Enteroviruses indeed developed two complementary aggregating strategies to enhance infectivity both upon entry into their host and during further propagation steps from one cell to another within the host. First, within the lumen of the host intestine, LPS from commensal bacteria aggregate polioviruses in biofilm-like structures, thus enhancing their attachment to host cells (Robinson et al., 2014). Second, within the host, enteroviral particles use another aggregation mechanism involving their release from infected cells in phosphatidyl serine lipid vesicles, as shown in Figure 40. These PS vesicles containing multiple viral genomes are very efficient at simultaneously transferring multiple viral genomes to an individual cell in a new round of infection, potentially facilitating genetic cooperation among diverse viral genomes (Chen et al., 2015).



**Figure 40. Clusters of enteroviruses.**

*Transmission electron micrographs of phosphatidyl serine vesicles containing numerous electron dense viral particles surrounded by an unilamellar membrane stained with anti-Annexin V immunogold labeling (asterisk). Scale bar: 100 nm. (from (Chen et al., 2015)).*

We are intimately convinced that similar examples of aggregating strategies developed by different viruses will progressively be unraveled within the next coming years. The formation of a viral biofilm may even be described for a virus unrelated to human lymphotropic retroviruses, opening new avenues of research on the evolutionary convergence or shared origin of this common property.

## General conclusion

Through a combination of microscopic approaches complemented with functional assays, the present work evidences that HIV-1 T-cell associated infectivity mostly resides at the cell surface in a viral biofilm. Enriched in ECM components, this biofilm represents a highly adhesive structure crucially contributing to the efficiency of HIV-1 cell-to-cell transmission between T cells and through DC-mediated *trans*-infection. Further, HIV-1 biofilm confers viral particles with an increased infectivity as compared to their cell-free counterparts, even in the presence of ArT and NAbs.

This work initiates the comprehensive description of the morphology and composition of HIV-1 biofilm as a new infectious entity and provides insights into its functional relevance. More specifically, our study provides some hints on the role of biofilm ECM composition in HIV-1 transmission as well as on the basis of increased infectivity. Simultaneously, our work arouses many new questions, among which the following are the more burning:

- Which mechanisms and molecular actors, both from cellular and viral origin, regulate HIV-1 biofilm formation?
- What is the precise nature of HIV-1 biofilm composition, both in ECM components and viral particles? How does this nature condition HIV-1 infectivity? Is HIV-1 biofilm architecture regulated and does it impact HIV-1 infectivity?
- As ArT and NAbs are not fully blocking HIV-1 biofilm-mediated transmission, is HIV-1 biofilm still present in ArT-treated patients?

Overall, a better understanding of the coevolution events regulating HIV-1 biofilm formation, composition and architecture is of prime importance to efficiently appraise this new infectious entity in the design of future therapeutical strategies.



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## **APPENDIXES**



## APPENDIX 1: T cell matrisome and HIV-1 infection

### 1. Modulated matrisome upon T cell activation

MatrisomeDB (updated August 2014 <http://matrisomeproject.mit.edu/other-resources/>) is a searchable database that provides live cross-referencing to gene and protein databases for every ECM and ECM-associated genes, integrating experimental proteomic data on ECM and ECM-associated proteins (Naba et al., 2012). As activation broadly reshapes T cell gene expression profiles, we browsed databases of modulated genes upon T cell activation in the search for matrisome using MatrisomeDB and present down-regulated and up-regulated matrisomes upon T cell activation in Table 5 and Table 6 resp.

**Table 5. Down-regulated matrisome upon T cell activation.**

Division	Category	Gene Symbol	Gene Name	Ref
Core matrisome	Collagens	Col1a2	collagen, type I, alpha 2	Teague
Core matrisome	ECM Glycoproteins	Igfbp4	insulin-like growth factor binding protein 4	Teague
Core matrisome	ECM Glycoproteins	Mgp	matrix Gla protein	Teague
Matrisome-associated	ECM Regulators	P4ha1	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide	Teague
Matrisome-associated	ECM-affiliated Proteins	LGALS3	lectin, galactoside-binding, soluble, 3	Howden
Matrisome-associated	ECM-affiliated Proteins	Sema4b	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4B	Teague
Matrisome-associated	Secreted Factors	CCL5	chemokine (C-C motif) ligand 5	Howden
Matrisome-associated	Secreted Factors	S100A9	S100 calcium binding protein A9	Howden
Matrisome-associated	Secreted Factors	Wnt1	wingless-related MMTV integration site 1	Teague

**Table 6. Up-regulated matrisome upon T cell activation.**

Division	Category	Gene Symbol	Gene Name	Ref
Core matrisome	Collagens	Col12a1	collagen, type XII, alpha 1	Teague
Core matrisome	Collagens	Col1a1	collagen, type I, alpha 1	Teague
Core matrisome	Proteoglycans	SRGN	serglycin	Howden
Matrisome-associated	ECM Regulators	Cela2a	chymotrypsin-like elastase family, member 2A	Teague
Matrisome-associated	ECM Regulators	Prss2	protease, serine, 2	Teague
Matrisome-associated	ECM-affiliated Proteins	Anxa2	annexin A2	Teague
Matrisome-associated	ECM-affiliated Proteins	Lgals3	lectin, galactose binding, soluble 3	Teague
Matrisome-associated	Secreted Factors	CSF2	colony stimulating factor 2 (granulocyte-macrophage)	Howden
Matrisome-associated	Secreted Factors	IFNG	interferon, gamma	Howden
Matrisome-associated	Secreted Factors	Ntf3	neurotrophin 3	Teague
Matrisome-associated	Secreted Factors	S100a4	S100 calcium binding protein A4	Teague
Matrisome-associated	Secreted Factors	S100a6	S100 calcium binding protein A6 (calcyclin)	Teague

Teague et al. activated T cells in C57BL/10 mice by intravenous injection of staphylococcal enterotoxin B (SEB). T cells were sorted out and gene expression was assessed 8 and 48 h after activation using Affymetrix gene arrays. Genes were considered down- (resp. up-) regulated when they displayed decreased (resp. increased) fold change at 8 and/or at 48h post-injection (Teague et al., 1999).

Howden et al. activated freshly isolated primary human CD4+ T cells with Phorbol 12-myristate 13-acetate (PMA) and ionomycin and identified modulated proteome at 2 and 4 h post-activation by nanoLC-MS/MS QuaNCAT, a conjunction of Bio-Orthogonal Non Canonical Amino acid Tagging (BONCAT) and Stable Isotope Labeling of Amino acids in Cell culture (SILAC). Genes were considered down- (resp. up-) regulated when they displayed decreased (resp. increased) fold change at 2 and/or at 4 h post-activation (Howden et al., 2013).

## 2. Modulated matrisome upon HIV-1 infection

HIV-1 infection deeply affects cellular gene expression profiles, both through direct gene modulation by viral factors and through the peculiar activation state it induces in T cells. Retrieving data collected from different systems exposed in Table 7, we undertook a comprehensive analysis of T cell matrisome modulated upon HIV-1 infection. We expose down-regulated and up-regulated matrisomes upon HIV-1 infection in Table 8 and Table 9 resp.

Strikingly, while no genes are common between down-regulated matrisomes upon T-cell activation and HIV-1 infection, only interferon  $\gamma$  IFN $\gamma$ , serglycin SRGN and collagen type 1 alpha 1 are common between both up-regulated matrisomes. These data support the concept that HIV-1 infection and T cell activation pathways exhibit a complex interplay.

**Table 7. Cellular systems and identification methods to analyse HIV-1 modulated transcriptome/proteome.**

<b>System</b>	<b>Gene identification method</b>	<b>Ref</b>
Inguinal lymph node biopsies from - untreated HIV-1-infected subjects at different clinical stages ( $n = 22$ ) - uninfected subjects ( $n = 5$ )	Whole-genome transcriptional analysis (Human Genome U133 Plus 2.0, Affymetrix)	(Li et al., 2009)
CD4(+) T cells from - HIV-1 infected individuals with a known date of seroconversion ( $n = 182$ ) - Elite Controllers ( $n = 16$ )	Whole-genome transcriptional profiling (Human-6 v3 Expression BeadChips, Illumina) → kept are genes correlating with a high viral load	(Rotger et al., 2010)
CD3(+) T cells from - progressors ( $n = 15$ ) - LTNP ( $n = 15$ )	Whole-genome transcriptional analysis (Agilent microarrays) → kept are genes differentially modulated between progressors and LTNP	(Salgado et al., 2011)
HLA-DR(-) CD4(+) T cells - HIV-1-infected persons treated with ArT ( $n = 14$ ) - Elite Controllers ( $n = 12$ ) - HIV-1-negative persons ( $n = 9$ )	Whole-genome transcriptional profiling (WG-DASL microarrays, Illumina) → kept are genes differentially modulated in infected persons and Elite Controllers with regard to non-infected subjects	(Vigneault et al., 2011)
CEMx174 cells non-infected or infected with HIV-1 LAI	Differential <b>proteome</b> analysis through $^{16}\text{O}/^{18}\text{O}$ labeling and quantitative LC-FTICR analyses	(Chan et al., 2007)

*FTICR = T Fourier transform ion cyclotron resonance mass spectrometer*

**Table 8. Down-regulated matrisome upon HIV-1 infection.**

Division	Category	Gene symbol	Gene name	Ref
Core matrisome	ECM Glycoproteins	COCH	coagulation factor C homolog, cochlin (Limulus polyphemus)	Li
Core matrisome	ECM Glycoproteins	CTGF	connective tissue growth factor	Li
Core matrisome	ECM Glycoproteins	DPT	dermatopontin	Li
Core matrisome	ECM Glycoproteins	FBLN1	fibulin 1	Li
Core matrisome	ECM Glycoproteins	HMCN1	hemicentin 1	Li
Core matrisome	ECM Glycoproteins	IGFBP3	insulin-like growth factor binding protein 3	Li
Core matrisome	ECM Glycoproteins	IGFBP5	insulin-like growth factor binding protein 5	Li
Core matrisome	ECM Glycoproteins	MATN4	matrilin 4	Chan
Core matrisome	ECM Glycoproteins	MGP	matrix Gla protein	Li
Core matrisome	ECM Glycoproteins	RELN	reelin	Li
Core matrisome	ECM Glycoproteins	SNED1	sushi, nidogen and EGF-like domains 1	Li
Core matrisome	ECM Glycoproteins	THBS1	thrombospondin 1	Li
Core matrisome	Proteoglycans	DCN	decorin	Li
Core matrisome	Proteoglycans	NCAN	neurocan	Chan, Rotger
Core matrisome	Proteoglycans	SPOCK2	sparc/osteonectin, ewcv and kazal-like domains proteoglycan (testican) 2	Li
Core matrisome	Proteoglycans	SPOCK3	sparc/osteonectin, ewcv and kazal-like domains proteoglycan (testican) 3	Li
Core matrisome	Proteoglycans	SRGN	serglycin	Li
Matrisome-associated	ECM Regulators	ADAM12	ADAM metallopeptidase domain 12	Li
Matrisome-associated	ECM Regulators	CTSB	cathepsin B	Chan
Matrisome-associated	ECM Regulators	CTSV	cathepsin L2	Chan
Matrisome-associated	ECM Regulators	F13A1	coagulation factor XIII, A1 polypeptide	Li
Matrisome-associated	ECM Regulators	MMP25	matrix metallopeptidase 25	Chan
Matrisome-associated	ECM Regulators	SERPIND1	serpin peptidase inhibitor, clade D (heparin cofactor), member 1	Chan
Matrisome-associated	ECM Regulators	TIMP3	TIMP metallopeptidase inhibitor 3	Li
Matrisome-associated	ECM-affiliated Proteins	ANXA7	annexin A7	Chan
Matrisome-associated	ECM-affiliated Proteins	CLEC4G	C-type lectin domain family 4, member G	Li
Matrisome-associated	ECM-affiliated Proteins	OVGP1	oviductal glycoprotein 1, 120kDa	Rotger
Matrisome-associated	ECM-affiliated Proteins	SDC1	syndecan 1	Li
Matrisome-associated	Secreted Factors	CXCL11	chemokine (C-X-C motif) ligand 11	Chan
Matrisome-associated	Secreted Factors	CXCL2	chemokine (C-X-C motif) ligand 2	Li
Matrisome-associated	Secreted Factors	IGF2	insulin-like growth factor 2 (somatomedin A)	Li
Matrisome-associated	Secreted Factors	MEGF6	multiple EGF-like-domains 6	Li
Matrisome-associated	Secreted Factors	S100A12	S100 calcium binding protein A12	Chan
Matrisome-associated	Secreted Factors	S100A5	S100 calcium binding protein A5	Chan
Matrisome-associated	Secreted Factors	THPO	thrombopoietin	Chan
Matrisome-associated	Secreted Factors	TNFSF11	tumor necrosis factor (ligand) superfamily, member 11	Li
Matrisome-associated	Secreted Factors	WNT7A	wingless-type MMTV integration site family, member 7A	Rotger

**Table 9. Up-regulated matrisome upon HIV-1 infection.**

Division	Category	Gene symbol	Gene name	Ref
Core matrisome	Collagens	COL1A1	collagen, type I, alpha 1	Vigneault
Core matrisome	ECM Glycoproteins	FBN3	fibrillin 3	Vigneault
Core matrisome	ECM Glycoproteins	FGL2	fibrinogen-like 2	Li, Rotger
Core matrisome	ECM Glycoproteins	FNDC7	fibronectin type III domain containing 7	Vigneault
Core matrisome	ECM Glycoproteins	IGFBP3	insulin-like growth factor binding protein 3	Vigneault
Core matrisome	ECM Glycoproteins	IGFBP4	insulin-like growth factor binding protein 4	Vigneault
Core matrisome	ECM Glycoproteins	LAMA5	laminin, alpha 5	Salgado
Core matrisome	ECM Glycoproteins	MATN1	matrilin 1, cartilage matrix protein	Vigneault
Core matrisome	ECM Glycoproteins	MFAP4	microfibrillar-associated protein 4	Vigneault
Core matrisome	ECM Glycoproteins	MMRN1	multimerin 1	Vigneault
Core matrisome	ECM Glycoproteins	PXDN	peroxidasin homolog (Drosophila)	Salgado
Core matrisome	ECM Glycoproteins	SPON2	spondin 2, extracellular matrix protein	Vigneault
Core matrisome	ECM Glycoproteins	THBS1	thrombospondin 1	Vigneault
Core matrisome	ECM Glycoproteins	VWA1	von Willebrand factor A domain containing 1	Chan, Vigneault
Core matrisome	ECM Glycoproteins	VWCE	von Willebrand factor C and EGF domains	Chan, Vigneault
Core matrisome	Proteoglycans	HSPG2	heparan sulfate proteoglycan 2	Vigneault
Core matrisome	Proteoglycans	PODN	podocan	Vigneault
Core matrisome	Proteoglycans	PRG2	proteoglycan 2, bone marrow (natural killer cell activator, eosinophil granule major basic protein)	Vigneault
Core matrisome	Proteoglycans	SRGN	serglycin	Vigneault
Matrisome-associated	ECM Regulators	A2M	alpha-2-macroglobulin	Vigneault
Matrisome-associated	ECM Regulators	ADAM9	ADAM metallopeptidase domain 9 (meltrin gamma)	Vigneault
Matrisome-associated	ECM Regulators	ADAMDEC1	ADAM-like, decysin 1	Li
Matrisome-associated	ECM Regulators	CST7	cystatin F (leukocystatin)	Li
Matrisome-associated	ECM Regulators	CSTL1	cystatin-like 1	Chan
Matrisome-associated	ECM Regulators	CTSG	cathepsin G	Vigneault
Matrisome-associated	ECM Regulators	EGLN3	egl nine homolog 3 (C. elegans)	Salgado
Matrisome-associated	ECM Regulators	FAM20C	family with sequence similarity 20, member C	Vigneault
Matrisome-associated	ECM Regulators	LOX	lysyl oxidase	Vigneault
Matrisome-associated	ECM Regulators	MMP28	matrix metallopeptidase 28	Vigneault
Matrisome-associated	ECM Regulators	MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	Vigneault
Matrisome-associated	ECM Regulators	SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	Li
Matrisome-associated	ECM Regulators	SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	Vigneault
Matrisome-associated	ECM Regulators	SERPINF1	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	Vigneault
Matrisome-associated	ECM Regulators	SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	Rotger
Matrisome-associated	ECM Regulators	SERPINI1	serpin peptidase inhibitor, clade I (neuroserpin), member 1	Vigneault
Matrisome-associated	ECM-affiliated Proteins	ANXA10	annexin A10	Chan
Matrisome-associated	ECM-affiliated Proteins	C1QB	complement component 1, q subcomponent, B chain	Li, Rotger
Matrisome-associated	ECM-affiliated Proteins	CLEC2B	C-type lectin domain family 2, member B	Li
Matrisome-associated	ECM-affiliated Proteins	CLEC7A	C-type lectin domain family 7, member A	Li, Vigneault
Matrisome-associated	ECM-affiliated Proteins	FCN1	ficolin (collagen/fibrinogen domain containing) 1	Li, Vigneault



Matrisome-associated	ECM-affiliated Proteins	FCN2	ficolin (collagen/fibrinogen domain containing lectin) 2 (hucolin)	Vigneault
Matrisome-associated	ECM-affiliated Proteins	GPC3	glypican 3	Vigneault
Matrisome-associated	ECM-affiliated Proteins	ITLN1	intelectin 1 (galactofuranose binding)	Vigneault
Matrisome-associated	ECM-affiliated Proteins	LMAN1L	lectin, mannose-binding, 1 like	Chan
Matrisome-associated	ECM-affiliated Proteins	REG1A	regenerating islet-derived 1 alpha	Vigneault
Matrisome-associated	ECM-affiliated Proteins	SDC4	syndecan 4	Vigneault
Matrisome-associated	Secreted Factors	BMP4	bone morphogenetic protein 4	Vigneault
Matrisome-associated	Secreted Factors	CCL3	chemokine (C-C motif) ligand 3	Vigneault
Matrisome-associated	Secreted Factors	CCL3L3	chemokine (C-C motif) ligand 3-like 3	Vigneault
Matrisome-associated	Secreted Factors	CCL4L1	chemokine (C-C motif) ligand 4-like 1	Vigneault
Matrisome-associated	Secreted Factors	CLCF1	cardiotrophin-like cytokine factor 1	Vigneault
Matrisome-associated	Secreted Factors	CXCL2	chemokine (C-X-C motif) ligand 2	Vigneault
Matrisome-associated	Secreted Factors	EPO	erythropoietin	Vigneault
Matrisome-associated	Secreted Factors	FASLG	Fas ligand (TNF superfamily, member 6)	Li
Matrisome-associated	Secreted Factors	FGF7	fibroblast growth factor 7 (keratinocyte growth factor)	Vigneault
Matrisome-associated	Secreted Factors	FGFBP1	fibroblast growth factor binding protein 1	Vigneault
Matrisome-associated	Secreted Factors	FGFBP2	fibroblast growth factor binding protein 2	Vigneault
Matrisome-associated	Secreted Factors	FGFBP3	fibroblast growth factor binding protein 3	Vigneault
Matrisome-associated	Secreted Factors	FSTL1	folliculin-like 1	Vigneault
Matrisome-associated	Secreted Factors	GDF3	growth differentiation factor 3	Chan
Matrisome-associated	Secreted Factors	IFNG	interferon, gamma	Li, Vigneault
Matrisome-associated	Secreted Factors	IL13	interleukin 13	Vigneault
Matrisome-associated	Secreted Factors	IL16	interleukin 16 (lymphocyte chemoattractant factor)	Vigneault
Matrisome-associated	Secreted Factors	IL17C	interleukin 17C	Vigneault
Matrisome-associated	Secreted Factors	IL18	interleukin 18 (interferon-gamma-inducing factor)	Li
Matrisome-associated	Secreted Factors	IL1B	interleukin 1, beta	Vigneault
Matrisome-associated	Secreted Factors	IL1RN	interleukin 1 receptor antagonist	Li
Matrisome-associated	Secreted Factors	IL2	interleukin 2	Vigneault
Matrisome-associated	Secreted Factors	IL6	interleukin 6 (interferon, beta 2)	Vigneault
Matrisome-associated	Secreted Factors	MDK	midkine (neurite growth-promoting factor 2)	Salgado
Matrisome-associated	Secreted Factors	NODAL	nodal homolog (mouse)	Vigneault
Matrisome-associated	Secreted Factors	PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	Salgado
Matrisome-associated	Secreted Factors	S100A8	S100 calcium binding protein A8	Li
Matrisome-associated	Secreted Factors	S100P	S100 calcium binding protein P	Vigneault
Matrisome-associated	Secreted Factors	TGFB1	transforming growth factor, beta 1	Vigneault
Matrisome-associated	Secreted Factors	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	Rotger
Matrisome-associated	Secreted Factors	TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b	Rotger
Matrisome-associated	Secreted Factors	TNFSF9	tumor necrosis factor (ligand) superfamily, member 9	Vigneault
Matrisome-associated	Secreted Factors	VEGFA	vascular endothelial growth factor A	Vigneault
Matrisome-associated	Secreted Factors	WNT1	wingless-type MMTV integration site family, member 1	Vigneault

### 3. Matrisome associated to HIV-1 virions

HIV-1 is known to incorporate or associate with a variety of host proteins upon viral particle assembly and budding. Retrieving data collected from viral preparations presented in Table 10, we browsed virion-associated proteomes in search for matrisome and expose hits in Table 11. Interestingly, while only the coagulation factor F13A1 was found down-regulated upon HIV-1 infection and associated to HIV-1 virions, 6 genes were found up-regulated by HIV-1 infection and associated to HIV-1 virions, namely an homolog of peroxidasin PXDN, heparin sulfate proteoglycan 2 HSPG2, alpha-2-macroglobulin A2M, the peptidase ADAM10, the inhibitor F1 of serpin peptidase SERPINF1 as well as the calcium binding protein S100A8.

**Table 10. Viral preparations used for identification of associated host proteins.**

<b>Virus</b>	<b>Cell line</b>	<b>Proteomics</b>	<b>Ref</b>
<b>NL4-3</b>	Hek	MS /MS	(Bregnard et al., 2013)
<b>NLAD8</b> (CD45-depleted)	MDM	microcapillary reversed-phase liquid chromatography-MS/MS ( $\mu$ RPLC-MS/MS)	(Chertova et al., 2006)
<b>SIV</b>	PBMCs from SM and RM	LC/MS/MS (common proteome to SM and RM)	(Stephenson et al., 2010)

*MDM = Monocyte derived macrophages, PBMCs = Peripheral Blood Mononuclear Cells, SM = Sooty mangabey, RM = Rhesus macaque, MS/MS = tandem Mass Spectrometry.*

**Table 11. Matrisome associated to HIV-1 and SIV virions.**

Division	Category	Gene Symbol	Gene Name	Ref
Core matrisome	Collagens	COL13A1	collagen, type XIII, alpha 1	Bregnard
Core matrisome	Collagens	COL28A1	collagen, type XXVIII, alpha 1	Bregnard
Core matrisome	ECM Glycoproteins	CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	Bregnard
Core matrisome	ECM Glycoproteins	COMP	cartilage oligomeric matrix protein	Stephenson
Core matrisome	ECM Glycoproteins	CYR61	cysteine-rich, angiogenic inducer, 61	Bregnard
Core matrisome	ECM Glycoproteins	EDIL3	EGF-like repeats and discoidin I-like domains 3	Bregnard
Core matrisome	ECM Glycoproteins	EMILIN2	elastin microfibril interfacier 2	Chertova
Core matrisome	ECM Glycoproteins	EMILIN3	elastin microfibril interfacier 3	Bregnard
Core matrisome	ECM Glycoproteins	FGG	fibrinogen gamma chain	Bregnard
Core matrisome	ECM Glycoproteins	FN1	fibronectin 1	Stephenson
Core matrisome	ECM Glycoproteins	LAMB1	laminin, beta 1	Stephenson
Core matrisome	ECM Glycoproteins	MFGE8	milk fat globule-EGF factor 8 protein	Bregnard
Core matrisome	ECM Glycoproteins	PXDN	peroxidasin homolog (Drosophila)	Bregnard
Core matrisome	ECM Glycoproteins	SLIT2	slit homolog 2 (Drosophila)	Bregnard
Core matrisome	ECM Glycoproteins	THBS4	thrombospondin 4	Stephenson
Core matrisome	ECM Glycoproteins	VTN	vitronectin	Bregnard, Stephenson
Core matrisome	Proteoglycans	BGN	biglycan	Stephenson
Core matrisome	Proteoglycans	FMOD	fibromodulin	Stephenson
Core matrisome	Proteoglycans	HSPG2	heparan sulfate proteoglycan 2	Stephenson
Core matrisome	Proteoglycans	VCAN	versican	Bregnard
Matrisome-associated	ECM Regulators	A2M	alpha-2-macroglobulin	Stephenson, Stephenson
Matrisome-associated	ECM Regulators	ADAM10	ADAM metallopeptidase domain 10	Bregnard
Matrisome-associated	ECM Regulators	ADAM15	ADAM metallopeptidase domain 15	Bregnard
Matrisome-associated	ECM Regulators	CSTB	cystatin B (stefin B)	Bregnard
Matrisome-associated	ECM Regulators	CTSD	cathepsin D	Bregnard
Matrisome-associated	ECM Regulators	F13A1	coagulation factor XIII, A1 polypeptide	Stephenson
Matrisome-associated	ECM Regulators	F2	coagulation factor II (thrombin)	Bregnard
Matrisome-associated	ECM Regulators	HABP2	hyaluronan binding protein 2	Stephenson
Matrisome-associated	ECM Regulators	HTRA1	HtrA serine peptidase 1	Bregnard
Matrisome-associated	ECM Regulators	ITIH2	inter-alpha (globulin) inhibitor H2	Chertova, Stephenson
Matrisome-associated	ECM Regulators	ITIH3	inter-alpha (globulin) inhibitor H3	Stephenson
Matrisome-associated	ECM Regulators	KNG1	kininogen 1	Bregnard
Matrisome-associated	ECM Regulators	LEPRE1	leucine proline-enriched proteoglycan (leprecan) 1	Bregnard
Matrisome-associated	ECM Regulators	MEP1A	meprin A, alpha (PABA peptide hydrolase)	Chertova
Matrisome-associated	ECM Regulators	MMP15	matrix metallopeptidase 15 (membrane-inserted)	Bregnard
Matrisome-associated	ECM Regulators	PLAT	plasminogen activator, tissue	Bregnard
Matrisome-associated	ECM Regulators	PLG	plasminogen	Bregnard
Matrisome-associated	ECM Regulators	PLOD1	procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1	Bregnard
Matrisome-associated	ECM Regulators	SERPINA4	serpin peptidase inhibitor, clade A (alpha-1 antipeptinase, antitrypsin),	Bregnard

Matrisome-associated	ECM Regulators	SERPINA4	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4	Bregnard
Matrisome-associated	ECM Regulators	SERPINA7	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	Chertova, Stephenson
Matrisome-associated	ECM Regulators	SERPINF1	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	Bregnard
Matrisome-associated	ECM Regulators	SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	Bregnard
Matrisome-associated	ECM Regulators	ST14	suppression of tumorigenicity 14 (colon carcinoma)	Chertova
Matrisome-associated	ECM-affiliated Proteins	ANXA1	annexin A1	Chertova
Matrisome-associated	ECM-affiliated Proteins	ANXA11	annexin A11	Bregnard, Stephenson
Matrisome-associated	ECM-affiliated Proteins	ANXA2	annexin A2	Bregnard
Matrisome-associated	ECM-affiliated Proteins	ANXA5	annexin A5	Bregnard
Matrisome-associated	ECM-affiliated Proteins	ANXA6	annexin A6	Bregnard
Matrisome-associated	ECM-affiliated Proteins	C1QTNF3	C1q and tumor necrosis factor related protein 3	Stephenson
Matrisome-associated	ECM-affiliated Proteins	CLEC11A	C-type lectin domain family 11, member A	Stephenson
Matrisome-associated	ECM-affiliated Proteins	CD44	CD44 molecule (Indian blood group)	Bregnard, Chertova, Stephenson
Matrisome-associated	ECM-affiliated Proteins	GPC1	glypican 1	Bregnard
Matrisome-associated	ECM-affiliated Proteins	GPC4	glypican 4	Bregnard
Matrisome-associated	ECM-affiliated Proteins	GPC6	glypican 6	Bregnard
Matrisome-associated	ECM-affiliated Proteins	LMAN1	lectin, mannose-binding, 1	Bregnard
Matrisome-associated	ECM-affiliated Proteins	PLXNB1	plexin B1	Bregnard
Matrisome-associated	ECM-affiliated Proteins	PLXNB2	plexin B2	Bregnard
Matrisome-associated	Secreted Factors	FLG2	filaggrin family member 2	Chertova
Matrisome-associated	Secreted Factors	S100A10	S100 calcium binding protein A10	Chertova
Matrisome-associated	Secreted Factors	S100A11	S100 calcium binding protein A11	Chertova
Matrisome-associated	Secreted Factors	S100A6	S100 calcium binding protein A6	Chertova
Matrisome-associated	Secreted Factors	S100A8	S100 calcium binding protein A8	Chertova
Matrisome-associated	Secreted Factors	TCHH	trichohyalin	Bregnard
Matrisome-associated	Secreted Factors	TNFSF14	tumor necrosis factor (ligand) superfamily, member 14	Chertova



## APPENDIX 2: Ongoing TransBioHIV cohort

Initiated in February 2014, the Biomedical Research Program TRANSBioHIV promoted by the French National Agency for AIDS and Hepatitis (ANRS) aims at recruiting 20 HIV-1 patients displaying a high viremia and CD4 counts reasonably high enough. As the rate of infected CD4+ T cells correlates with the viremia and inversely correlates with total CD4 counts (see INTRODUCTION I.2.b. Symptoms and stages of the disease), those criteria are meant to optimize the probability to isolate infected CD4+ T cells from blood samples. In order to assess the effect of anti-retroviral treatment on biofilm formation, two sample collections are planned: one at the initiation of HAART and another one after 3 months under treatment. So far, six patients have been successfully included and their clinical characteristics are given in the following table.

**Table 12. Clinical characteristics of HIV-1 patients from the TransBioHIV cohort.**

Patient	Sex	Age	Treated since (weeks)	M0		M3		Treatment
				CD4 count	RNA copies	CD4 count	RNA copies	
01	M	30	4	426*	25,547*	No sample		FTC + DF + Rilpivirine
02	M	54	8	226	83,780	280**	232**	?
03	M	48	1	70	189,980	141***	<40***	2 INTI + Raltegravir
04	M	27	0	456°	41,450°	784	417	ABC + 3TC + Rilpivirine
05	M	35	0	782#	37,490#	434***	784***	FTC + DF + Rilpivirine
06	M	28	0	626°	37,484°	678***	347***	FTC + DF + Rilpivirine

*CD4 counts and RNA copies are usually given the day the sample was taken except*

*\*: 3 weeks before sample taken*

*\*\* : 3 months before*

*\*\*\* : 2 months before*

*° : 1 month before*

*# : 6 months before*



## APPENDIX 3: Correlative confocal/cryoSEM

A method manuscript describing our correlative confocal/cryoSEM approach is currently in preparation. We give its running title, authors' contribution as well as an initial abstract. The technique and its advantages are described in details below.

### **Novel cryo-SEM correlative approach for double immunolocalization in viral biofilms**

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PIs: Maria-Isabel Thoulouze, Ana-Sartori Rupp\*.

\* = PFMU, Institut Pasteur.

*We describe a novel correlative light/cryo-scanning electron microscopy method to perform high resolution imaging & multiple immuno-localisation of target proteins in viral biofilms. Surface proteins of interest, immuno-labeled with a mixture of fluorescent and gold conjugated antibodies, are imaged in 3D by confocal microscopy and further recovered and imaged by cryo-scanning electron microscopy. In this method, we uniquely combine a cryogenic approach that guarantees the structural preservation of sensitive structures with a correlative approach that ensures the specificity of target proteins immunolocalisation.*

HIV-1 biofilm ECM components form a jelly-like structure that is extremely sensitive to the use of classic electron microscopy methods involving dehydration. In the literature, bacterial and fungal biofilms have been successfully characterized in their hydrated state by cryo-SEM (Alhede et al., 2012; Villena et al., 2010), where the sample is kept in its (native) hydrated state before freezing, thus ensuring the highest level of structural preservation. Cryo-SEM provides high-resolution information on biofilm ultrastructural details and on its cellular environment (Alhede et al., 2012). In addition, cryo-SEM is compatible with immunodetection of surface proteins (Erlandsen et al., 2001).

Confocal laser scanning microscopy (CLSM) is a powerful tool to identify biofilm-producing cells and their enrichment in ECM proteins. Ideally, information coming from CLSM and cryoSEM can be combined by using a correlative light electron microscopy



approach, thus linking fluorescence characterization and high resolution structural information on the same sample (Alhede et al., 2012).

Here we propose a novel correlative light/cryo-SEM (CL/cryo-SEM) approach in combination with simultaneous immunofluorescence and immunogold detection of proteins in cryogenic conditions that allows the characterization of the surface ultrastructure of hydrated infectious biofilms in link with their composition in ECM proteins. To our knowledge, there are no home-made or commercial solutions published until now. We develop a flexible home-made method, that is applicable to isolated cells on glass coverslips and that is adaptable to any CLSM and SEM imaging systems.

HIV-1 infected cells are PFA-fixed on MatTek Petri dishes containing the CELLocate glass bottom coverslip. The CELLocate cover slip consists of an etched grid with coordinates that allow to easily identify cell positions of interest in RT-CLSM and RT-SEM (Mironov and Beznoussenko, 2012).

Viral biofilms are then detected by simultaneous labeling of HIV-1 envelope (env) and biofilm ECM components (in our examples we labeled agrin and collagen). Fluorescence triple labeling arising from HIV-1 Env, agrin and collagen enables the localization the CLEM area of interest containing biofilm-producing cells.

If only fluo-labeling is performed, the diffraction-limited resolution of CLSM (~200 nm) determines the spatial accuracy with which the structures are recovered in the SEM unless the proteins are localized with high resolution by immunogold labeling. Thus, in order to gain more insight into the precise localization of Env and agrin proteins, we develop a strategy based on the use of a mixture of two distinct secondary antibodies coupled to a fluorescent tag and to a gold particle. More precisely, we label HIV-1 Env with a mouse primary antibody detected with two secondary antibodies, one coupled to Cy3 and the other to 10 nm gold particles. Primary rabbit antibody to agrin is detected using a secondary antibody coupled to Cy5 and a protein A-gold (pAg) coupled to 20 nm gold particles. Specificity of the staining and absence of cross-interactions were controlled through labeling of each antigen either by the fluorescently-coupled antibody or by the antibody coupled to the gold nanoparticle alone.

Biofilm producing cells are first imaged in 3D by CLSM. As CELLocate marks are obscured by a layer of ice in cryo-SEM, we manually mark with a diamond-scriber a “T” shaped sign above the correlative positions, far enough from the cells of interest to not

endanger them. The sample is then frozen in its hydrated state in slush Nitrogen and transferred via a cryo-transfer unit to the SEM.

In the SEM, we can recover and image at low magnification the “T” mark and identify the position (the coordinates) of the selected cells. Normally in SEM, the surface ultrastructure is imaged using SE detectors. However, in cryo-conditions, the morphology of HIV-1 biofilms is better revealed by a backscattered electrons detector (BSE) that is highly directional and detects subtle topographical differences. In BSE detection, electrons penetrate 500-1000 nm below the sample surface and the signal is sensitive to the atomic number Z of the elements composing the sample (chemical contrast generation). BSE detection simultaneously enables to localize with higher resolution the immunogold-labeled proteins of interest within the biofilm.

This experiment has been performed twice and will be repeated soon in order to confirm our observations. From these first two attempts, we can already draw several conclusions. First, the correlative process is successful and enables to image the same biofilms both in CLSM and cryo-CLEM. Second, by using a BSE detector, we succeeded in detecting the first layer of virions embedded and covered by the jelly-like meshwork of biofilm ECM. In addition, BSE detector enabled the detection and localization of gold labeling.

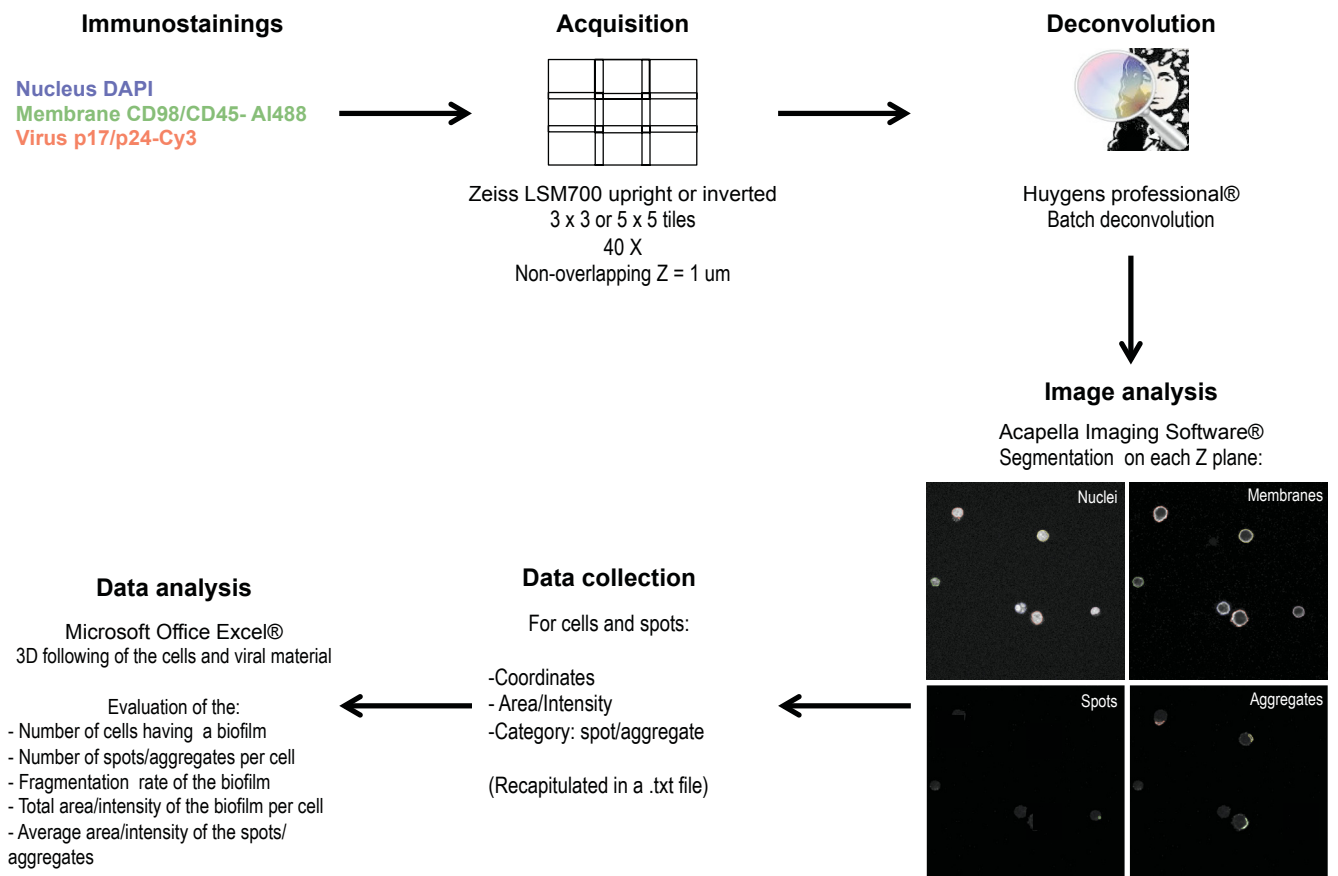
From CLSM observations, we can confirm that both antigens are accessible to secondary antibodies coupled to a fluorescent molecule. Interestingly, the corresponding immunogold labeling shows differences in terms of accessibility for room-temperature (RT) and cryo-CLEM approaches. In cryo-SEM, we so far detected only surface labeling of biofilm matrix components (20 nm gold particles), and no gold labeling for the viral envelope (10 nm gold particles) whereas both are well detected at RT. These discrepancies have two possible explanations. First, it is possible that the brightness of the biofilm observed in cryo conditions masks the 10 nm gold particles that are less bright than the 20 nm ones. Second, we can also envision the possibility that Env antigens are more deeply embedded in HIV-1 biofilm. 10 nm gold particles would hence be masked by the mesh of ECM proteins preserved in cryo-conditions. In RT conditions, the integrity of the biofilm matrix is altered by dehydration, leaving us with the advantage of exposing the 10 nm gold particles. Envelope 10 nm gold immunolabeling then becomes more accessible for detection but to the expense of the biofilm structural preservation.

In order to discriminate between those two hypotheses, we plan to use larger gold particles to detect Env labeling. If those larger particles are still observed in RT-SEM and still not in cryo-SEM, it would mean that they still reach anti-Env primary antibodies within HIV-1 biofilm but that the gold particles are masked by the ECM preserved in cryo conditions.

Overall, we report here the first use of correlative CLSM/cryo-SEM combined to a double labeling both in fluorescence and immunogold. This technique enabled us to describe HIV-1 biofilm as a potential protective microenvironment for HIV-1 viral particles it contains.

## APPENDIX 4: Automated quantification of the biofilm on confocal images

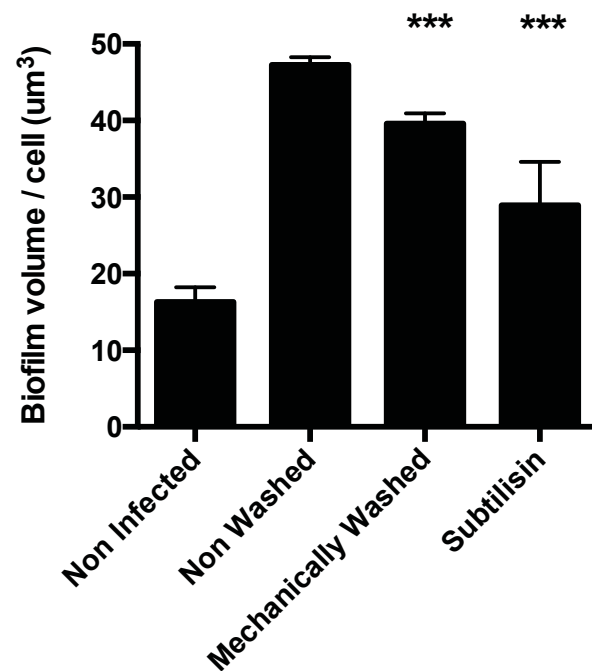
With the aim to analyze the correlation between the efficiency of HIV-1 cell-to-cell transfer and the morphology of HIV-1 biofilm, we developed the following workflow based on the automated quantification of confocal images, as depicted in Figure 41.



**Figure 41. Workflow for automated quantification on confocal images.**

*Our quantification workflow uses nucleus, membrane and HIV-1 MAp17 or CAp24 immunostainings as entry parameters. Following semi-automated acquisition of mosaics on a Zeiss LSM700 confocal microscope, images are deconvoluted and analyzed using an Acapella® script developed with the help of Dr Anne Danckaert (PFID, Institut Pasteur). Biofilms in the shape of MAp17 or CAp24 aggregates or spots are segmented and associated to their carrying cell detected thanks to the nucleus and membrane stainings. Data are collected and analyzed using an home-made Excel macro giving quantifications and morphological characteristics of the biofilm as outputs.*

When applied to ACH2 cells subjected to 2 to 3 Mechanical washes or treated with Subtilisin, this quantification measures 20 and 50% reduction in the volume of the biofilm resp (Figure 42). As a background volume is detected in Non Infected cells, this result would requires the implementation of intensity and fragmentation parameters, as well as the fraction of positive cells to give a comprehensive picture of biofilm morphological modifications induced by Mechanical washes and Subtilisin treatments. Should this automated quantification be reliable, its use could be extended to Collagenase and Heparin treatments that obviously alter biofilm architecture and organization.



**Figure 42. Automated quantification of the volume of HIV-1 biofilm.**

*ACH2 cells were left Non washed or subjected to Mechanical washes or Subtilisin treatment. Cells were surface-stained for the plasma membrane (CD98) and stained in permeabilizing conditions for HIV-1 MAb17 in the presence of DAPI. Shown are mean + SEM of biofilm volumes quantified on confocal images for at least 50 cells from 4 independent experiment. Non infected cells are shown as controls.*



## Résumé

La dissémination du VIH-1 par contacts cellulaires est plus efficace que sa transmission par particules virales libres. Cependant, la nature du matériel infectieux transféré à la jonction reste très mal connue.

Nos travaux révèlent que l'infectivité du VIH-1 associée aux lymphocytes T est majoritairement portée à la surface cellulaire dans un biofilm viral. Initialement décrit pour le rétrovirus lymphotrope HTLV-1 (Human T-cell Leukemia Virus type-1), le biofilm viral est une colonie extracellulaire de particules virales infectieuses enchâssées dans un cocon de matrice extracellulaire (MEC). Par un panel de techniques de microscopie, nous décrivons la présence de biofilms viraux à la surface de lymphocytes T infectés par le VIH-1 (lignées chroniquement infectées, lymphocytes CD4<sup>+</sup> primaires infectés *in vitro* par des souches de laboratoire et des isolats primaires, lymphocytes de patients). Nous identifions certains éléments de la MEC enrichis dans le biofilm du VIH-1 et démontrons que certains de ces composants, modulés par l'infection, favorisent la transmission du VIH-1. En effet, le biofilm viral joue un rôle clé dans la transmission directe (entre lymphocytes T) et indirecte (trans-infection) du VIH-1. De plus, le biofilm du VIH-1 confère une infectivité accrue aux particules virales, avantage préservé en présence d'antirétroviraux et d'anticorps neutralisants.

L'ensemble de notre travail identifie une nouvelle entité infectieuse cruciale pour la dissémination du VIH-1 par contacts cellulaires. Ce nouveau mécanisme de transmission, potentiellement généralisable à d'autres virus, sera désormais à prendre en compte dans l'élaboration de nouvelles stratégies thérapeutiques.

## Abstract

HIV-1 cell-to-cell spread is thousands fold more efficient than cell-free infection; yet the nature of the infectious material transferred at the junction remains poorly documented.

We found that HIV-1 T cell-associated infectivity mostly resides at the cell surface in a viral biofilm. Initially described for HTLV-1, a viral biofilm is defined as extracellular viral particles aggregated within a scaffold of extracellular matrix (ECM) components exposed at the surface of infected cells. Using a combination of microscopy techniques, we report the presence of HIV-1 biofilms at the surface of HIV-1 infected T cells (chronically infected T cells lines as well as primary CD4<sup>+</sup> T cells infected *in vitro* with HIV-1 laboratory strains as well as primary isolates). Importantly, we show that CD4<sup>+</sup> T cells isolated from HIV-1 patients produce a viral biofilm as well. We partially characterize the composition of HIV-1 biofilm in ECM components and unravel their contribution to HIV-1 transmission. We show that HIV-1 biofilm is transferred both between T cells and during dendritic-cell (DC)-mediated *trans*-infection and confers viral particles with an increased infectivity as compared to their cell-free counterparts. This increased infectivity is preserved in the presence of antiretroviral treatment and neutralizing antibodies.

Our findings hence identify HIV-1 biofilm as a new infectious entity central for the efficiency of HIV-1 cell-to-cell transmission. This new mechanism of intercellular transmission might be shared by other viruses and will require appraisal in the design of future therapeutical strategies.