Faculdade de Engenharia da Universidade do Porto

Structure and molecular characterization of HTLV-1 biofilm: impact on viral assembly and transmission

Maria João Tavares Sousa

DISSERTATION

MESTRADO INTEGRADO EM BIOENGENHARIA – BIOTECNOLOGIA MOLECULAR

Supervisor: Hélène Dutartre, PhD, INSERM[1] & CIRI[2]

Co-supervisor: Manuel Vilanova, PhD, i3s[3] & ICBAS[4]

June 2021

[1] Institut National de la Santé et de la Recherche Médicale
[2] Centre International de Recherche en Infectiologie
[3] Instituto de Investigação e Inovação em Saúde
[4] Instituto de Ciências Biomédicas Abel Salazar

Structure and molecular characterization of HTLV-1 biofilm: impact on viral assembly and transmission

Maria João Tavares Sousa

MESTRADO INTEGRADO EM BIOENGENHARIA – BIOTECNOLOGIA MOLECULAR

June 2021

i. Abstract

The *Retroviridae* family can be defined by a type of viruses with RNA that insert a copy of their genome into the DNA of a host cell. Human T-cell leukaemia virus type 1 (HTLV-1) is a retrovirus which is mainly transmitted by breastfeeding or sexual contact between humans. The first detection and isolation of HTLV-1 was in 1979 and its origin in humans occurred due to the transmission, from primates to humans, of the Simian T-cell leukaemia virus type 1 (STLV-1). Two years after, another virus was identified as member of the HTLV family. However, soon after, it appeared that it was a different virus which they named as Human Immuno defiency virus (HIV). Although being the first human oncogenic retrovirus, HTLV became a neglected virus since the discovery of HIV. Accordingly, because of their high similarity at both structural and protein levels, most HTLV studies are performed in light of discoveries done in HIV.

HTLV-1 infected 15 to 20 million people worldwide. However, HTLV-1 infection remains asymptomatic in most of the carriers, with only 5-10% of the infected individual experiencing morbidity and mortality due to HTLV-1. In most of the pathological cases, the infected individuals develop an aggressive haematological malignancy called adult T cell leukaemia/lymphoma (ATLL) or an incapacitating neuroinflammatory disorder named tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM).

Although HTLV-1 infects mainly CD4+ T cells, it has tropism for many cell types, but the mechanism of its dissemination remains unclear. Dendritic cells (DCs) are abundant at the digestive and sexual mucosa unlike T cells. Knowing that these mucosae are the entry routes of HTLV-1 infection, DCs might be the first cell type encountered by HTLV-1, inasmuch as these cells were demonstrated to be infected and are more susceptible than T-lymphocytes to HTLV-1, at least *in vitro*. DCs have an important immunologic role as antigen-presenting cells able to shape specific adaptive immune responses. In addition, because HTLV-1 viral particles are not infectious, and because HTLV-1 spread needs close cell-cell contact, it was hypothesized that T-cells might be infected by cell-cell contact with DCs during the immune response. Three viral particle transfer pathways using cell-cell contact have been described for HTLV-1: (i) virological synapse, (ii) the formation of intercellular conducts, and (iii) the production of adhesive extracellular aggregates, or viral biofilms, described at the surface of infected T lymphocytes. Biofilms are common in microbiology, but until recently were mostly associated with Bacteria and less often with Fungi. However, nowadays, it is known that viruses can also be linked with biofilms. Although HTLV-1 is the only virus described to be associated with this structure, more and more evidences suggest that other viruses, and specially HIV, might also have the ability to form viral biofilms.

However, how the viral biofilm is formed and what is its role in viral transmission during cell-cell contact is not fully understood.

In this work, we studied the role of two cellular membrane proteins involved in vesicular trafficking: Caveolin-1 and SNAP25, that were also shown to be important for HIV-1 cycle or spreading. Interestingly, the expression of these proteins is upregulated in HTLV-1 infected cells. We thus hypothesized that Caveolin-1 and/or SNAP25 might be important for the retention of retroviruses in viral biofilm at the surface of infected cells. We performed several independent

silencing experiments using two cell lines. The silencing was acquired using the technology of shRNA carried by lentivectors. Thus, we observed that while Caveolin-1 silencing increased both p19 expression and viral transmission it did not change the level of virus at the surface of infected cells. In contrast, SNAP25 silencing decreased viral transmission, as well as p19 cytoplasmic level but not surface viral protein expression nor biofilm distribution.

To conclude, with this work we demonstrated that Caveolin-1 restrict viral transmission, while SNAP25 is crucial for viral transmission but none of them seemed to have an effect on the density of viral particles at the surface's biofilm. However, more studies need to be done in order to understand the role of these proteins and others within the viral biofilm.

ii. Resumo

A família *Retroviridae* é composta por vírus de RNA capazes de inserir uma cópia do seu genoma no DNA de uma célula hospedeira. O vírus linfotrópico da célula T humana (HTLV em inglês) é um retrovírus transmitido principalmente por amamentação e/ou contacto sexual. Este vírus foi detetado em humanos e isolado pela primeira vez em 1979. A sua origem deu-se pela transmissão, dos primatas para os humanos, do vírus linfotrópico das células T símio.

Dois anos após descoberta do HTLV, outro retrovírus foi descrito como sendo um novo membro da família do HTLV. Contudo, pouco tempo depois, os cientistas perceberam que se tratava de um vírus diferente, o qual foi denominado por vírus da Imunodeficiência Humana (VIH).

Apesar do HTLV ter sido o primeiro retrovírus oncogénico isolado de humanos, este tem sido altamente negligenciado desde a descoberta do VIH. Por outro lado, a elevada semelhança, tanto a nível estrutural como proteico, entre estes dois vírus tem permitido o estudo do HTLV baseado nas descobertas e no conhecimento existente sobre o VIH.

Atualmente, o HTLV infetou cerca de 15 a 20 milhões de pessoas. Todavia, a infeção causada por este vírus permanece assintomática na maioria dos seus portadores, sendo que apenas 5-10% dos indivíduos infetados desenvolvem uma patologia. Na maioria dos casos patológicos, os indivíduos desenvolvem uma malignidade hematológica agressiva denominada por leucemia/linfoma das células T adultas ou uma doença neuroinflamatória incapacitante denominada por paraparesia espástica tropical/mielopatia associada ao HTLV. Ambas estão associadas a elevados índices de mortalidade.

Embora o HTLV-1 infete principalmente os linfócitos T CD4+, este vírus tem tropismo para muitos tipos de células; contudo, o mecanismo de disseminação ainda não é completamente conhecido. As células dendríticas são abundantes tanto na mucosa digestiva como sexual, ao contrário das células T. Sabendo que estas são as vias de entrada do HTLV no organismo, é de se supor que as células dendríticas sejam o primeiro tipo de células a encontrar este vírus, na medida em que já foi demonstrado *in vitro* que estas células podem ser infetadas e são mais suscetíveis do que os linfócitos T ao HTLV-1. As células dendríticas têm um papel imunológico importante como células apresentadoras de antigénios capazes de moldar respostas imunitárias adaptativas especificas. Desta forma, foi levantada a hipótese de que os linfócitos T CD4+ eram então infetados pelas células dendríticas durante uma resposta imunitária. Esta hipótese assenta no facto das partículas virais do HTLV não serem infeciosas e da propagação do HTLV requerer o contacto próximo entre células.

Atualmente, foram descritas três vias de transferência de partículas do HTLV através do contacto entre duas células: (i) sinapse virológica, (ii) a formação de ductos intercelulares, e (iii) a produção de agregados extracelulares adesivos, ou biofilmes virais, descritos na superfície dos linfócitos T infetados. Os biofilmes são comuns em microbiologia, mas até há pouco tempo estavam, sobretudo, associados a bactérias e até a fungos. No entanto, atualmente, sabe-se que os biofilmes podem ser dependentes de infeções por vírus. Embora o HTLV seja o único vírus descrito que esteja associado a este tipo de estrutura, cada vez mais é proposta a existência de biofilmes para outros vírus, tais como o VIH. Porém, a génese do biofilme viral e o seu papel na transmissão do HTLV durante o contacto celular não são ainda totalmente compreendidas.

Desta forma, neste trabalho estudou-se o papel de duas proteínas membranares envolvidas no tráfico vesicular, a Caveolina-1 e SNAP25, que demonstraram ser importantes para o ciclo de vida e propagação do VIH-1. Curiosamente, a expressão destas proteínas é amplificada em células infetadas pelo HTLV-1, o que sugere que tanto a Caveolina-1 como a proteina SNAP25 podem ser importantes para a retenção do retrovírus no biofilme viral à superfície das células infectas.

Assim, realizámos várias experiências independentes de silenciamento baseado na tecnologia de shRNA transportados por lentivetores. Este silenciamento ocorreu de forma independente para as duas proteínas membranares e em duas linhas celulares diferentes. Observamos então que, enquanto o silenciamento da proteína Caveolina-1 promoveu o aumento tanto da expressão de p19 como da transmissão viral, este não alterou o nível de vírus na superfície das células infetadas. Pelo contrário, o silenciamento da proteína SNAP25 diminuiu a transmissão viral, bem como o grau de p19 citoplasmático, mas não alterou a expressão viral superficial.

Em suma, com este trabalho demonstrados que a proteína Caveolina-1 restringe a transmissão viral, enquanto a proteína SNAP25 é crucial para a transmissão do HTLV-1. Nenhuma destas proteínas parece ter efeito sobre a densidade de partículas virais no biofilme à superfície das células.

iii. Acknowledgements

Em primeiro lugar, nada disto seria possível sem o apoio fundamental da minha família. Por isso, em primeiro lugar, gostaria de fazer um agradecimento muito especial à minha mãe, ao meu pai, à minha irmã, aos meus tios e avós, que sempre me incentivaram a ser uma mulher independente. Sem nunca me forçarem a fazer nada, sempre me apoiaram em todas as escolhas que tomei. Muito obrigada pela confiança que têm em mim. Eu sou-vos muito grata por todo o amor e carinho que me deram.

I would like to thank my supervisor Dr. Hélène Dutartre for giving me the opportunity to do research and providing guidance thought-out this work. I am very thankful for the cooperation, knowledge, and availability to assist me in this work. Thank you for everything. It is very good to work under your orientation. Eu também gostaria de agradecer ao professor Dr. Manuel Vilanova, por ter aceitado ser o meu coorientador. Muito obrigada por estar disponível para me auxiliar neste trabalho.

I also have to thank all the members of the host team. An amazing team, where I really felt integrated. Dr. Chloé Journo, thank you for all the help and guidance giving. My special thanks to Auriane. You know I am very thankful for your great patience at every moment that I was stressed and for all that you teach me. More than that, thank you for your friendship. Samuel, thank you for all the help you gave me, and for always making me laugh. Welcome to the biofilm group! It is amazing to work near and with you two! Karim, thank you for doing me company during the IF analysis. I am very happy to have met you. Julien, Janelle and Sandrine, thank you so much for all the help you gave me.

Também gostaria de fazer um agradecimento às pessoas muito especiais que estiveram comigo nestes 5 anos. Em primeiro lugar, Cat, muito obrigada por todas a horas passadas ao telefone a falar de *tudo-e-nada* e por estares sempre presente nos momentos mais importantes. Sofia, muito obrigada por todas as conversas profundas que tivemos desde o início e ao mesmo tempo por seres uma pessoa muito animada. Sempre adorámos ouvir sobre as tuas aventuras. Bia, muito obrigada por seres uma pessoa tão boa, por seres uma excelente conselheira e muito obrigada por estares sempre pronta para a diversão. Margarida, os últimos anos não seriam a mesma coisa sem ti. Muito obrigada por todos os conselhos quando precisava de tomar decisões importantes. Sou muito grata por te ter conhecido. E por fim, Rui (*Lástima*), tenho pena de não termos feitos mais trabalhos juntos. Apesar de tudo, fazíamos uma excelente dupla. E muito obrigada por me aturares quando estou stressada.

I would like to thank my boyfriend for always being by my side. Yema, you are the best listener and advisor I could have. Blessed the day I went to that Salsa class. Since then, I am a happier and smiling person. Thank you so much for everything.. Ah, that's right! Get ready to listen to the defense of this dissertation at least 2 (x10) times.

Por fim, queria agradecer ao senhor da residência que me colocou num quarto com varanda. Foi muito difícil redigir este documento com este calor.

"The future belongs to those who believe in the beauty of their dreams."
-Eleanor Roosevelt

iv. Contents

v. List of Figures

vi. List of Tables

vii. List of Abbreviations

A

AIDS	Acquired Immunodeficiency Syndrome
ATLL	Adult T-cell Leukaemia/Lymphoma
AZT	Zidovudine

B

BM	Bone Marrow

C

CA	Capsid
CBP	CREB Binding Protein
CCR4	CC Receptor 4
CREB	Cyclic AMP Response Element Binding protein
CTD	C-terminal Domain
CXCL10	C-X-C motif Chemokine Ligand 10

D

DCs	Dendritic cells
dsDNA	Double-standed DNA

G

GLUT-1	Glucose-transporter 1

H

HAU	HTLV-1 Associated Uveitis
HBZ	HTLV-1 Basic Zipper/ Haemoglobin Subunit Zeta Protein
HIV-1	Immunodeficiency Virus type 1
HIV-2	Immunodeficiency Virus type 2
HSCs	Hematopoietic Stem Cells
HSPG	Heparan Sulphate Proteoglycan
HTLV-1	Human T-cell Leukaemia Virus type 1 / Human T-Lymphotropic Virus type 1

I

ICAM-1	Intercellular Adhesion Molecule
IFN-γ	Interferon gamma
IFNs	Interferons
IL-10	Interleukin-1
IN	Integrase
INF-α	Interferon alpha
INF-β	Interferon beta

L

LFA-1	Lymphocyte Function-associated Antigen
LTR	Long Terminal Repeat

M

MA	Matrix

MDDCs	Monocytes Derived Dendritic Cells
MTOC	Microtubule Organization Center

N

NC	Nucleocapsid
NF-kB	Nuclear Factor kB
NHP	Non-human primate
NRP-1	Neuropilin Receptor 1
NTD	Amino-terminal Domain

O

ORF	Open Reading Frame

P

pDCs	Plasmacytoid Dendritic Cells
PKR	Protein Kinase R
PM	Plasma membrane
Pro	Viral Protease
PVL	Proviral Load

R

RexRE	Rex-responsive Element
RH	RNase H
RSV	Rous Sarcoma Virus
RT	Reverse Transcriptase

S

SAMHD1	SAM domain and HD Domain-containing protein 1
SEM	Scanning Microscopy
SIV	Simian Immunodeficiency Virus
SNAP25	Synaptosomal-associated Protein of 25 kDa
STLV-1	Simian T-lymphotropic Virus Type 1
STX4	Syntaxin-4 protein
SU	Surface Unit

T

TGF-β	Transforming Growth factor-beta
TLR7	Toll-like Receptor 7
TM	Transmembrane Unit
TRE	Tax-response Element
TSP/HAM	Tropical Spastic Paraparesis/HTLV-1 Associated Myelopathy

V

VEGF	Vascular Endothelium Growth Factor
vRNA	Viral genomic RNA
VS	Virological Synapse

viii. Document Structure

This work is structured as followed: **Chapter 1** contextualizes the theme, exposing some associated problems and presenting the objectives of this work; **Chapter 2** describes the important aspects of both viral tropism and replicative cycle of HTLV-1; **Chapter 3** reviews the viral biofilm and related studies; **Chapter 4** presents the material and methods used during the execution of this work; **Chapter 5** and **Chapter 6** present the results obtained and discussion of these, respectively. At the end it is presented the most important conclusions and presented prospects for future work.

Chapter 1

Introduction

1.1. Epidemiology

During the last decades, the contact between different animals' species and humans, allowed viral cross-species transmissions, which have been considered the main source of the biggest viral epidemics, such as Ebola, flu and Covid-19 [1, 2].

Non-human primates (NHPs) are genetically and physiologically similar to humans, so some simian viruses can represent a risk for the human health. As an example, it is now well demonstrated that human immunodeficiency virus type 1 and 2 (HIV-1/2) are linked to several independent interspecies transmissions of diverse types of simian immunodeficiency viruses (SIV) from African monkeys and chimpanzees. Moreover, human T-cell leukaemia virus type 1 or human T-lymphotropic virus type 1 (HTLV-1) has a similar history: the origin of HTLV-1 is a result of interspecies transmission of simian T-lymphotropic virus type 1 (STLV-1)[1], with still ongoing episodes of STLV transmission to humans [3].

In 1979, HTLV-1 was identified for the first time in a T-cell line from a patient with cutaneous T-cell lymphoma, and later in a patient with adult T-cell leukaemia [4]. This discovery was the first formal proof that human retroviruses exist and suggested their etiological role in human cancer, a hypothesis that had been proposed decades before. Nowadays, it is known that HTLV-1 is directly associated with two aggressive T-cell malignancies: (1) adult T-cell leukaemia/lymphoma (ATLL) and a progressive neurological condition, (2) tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM) [1].

The first epidemiologic studies demonstrated that South-western Japan was the highest endemic area for the virus. Further studies reported that Caribbean and many African countries were also endemic zones. Nowadays, it is recognized that the South-western part of Japan, Australo-Melanesia, Caribbean, Latin America, sub-Saharan Africa, and foci in the Middle East are the most endemic regions of HTLV-1(**Figure 1**) [4].

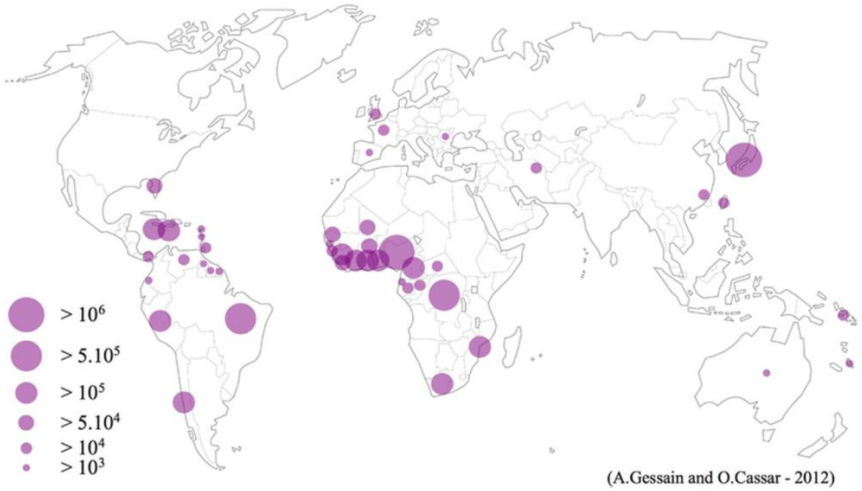


Figure 1 - Geographic distribution of the main foci of HTLV-1. HTLV-1 endemic areas are the South-western part of Japan, Australo-Melanesia, Caribbean, Latin America, sub-Saharan Africa, and foci in the Middle East. Image retrieved from Gessain A. & Cassar O. Frontiers in Microbiology 2012 [5].

Besides HTLV-1, which is the most pathogenic form for humans, there are three more subtypes of HTLV: HTLV-2, HTLV-3 and HTLV-4. HTLV-2 was the second human retrovirus described. This subtype is associated with a mild neurological disease, and it has a worldwide prevalence. While HTLV-1 have main tropism for CD4+ T-cells, HTLV-2 prefers CD8+ T-cells. Clonal expansion[1] seems to be much more accentuated in CD4+ infected T-cells than in CD8+ cells, which could explain the lower pathogenicity of HTLV-2 [6]. HTLV-3 and HTLV-4, on the other hand, were identified only in Africa [7]. HTLV-3 was first isolated from a male pygmy and HTLV-4 was isolated from a man who was bitten by a NHP during hunting activities [1]. HTLV-3 and HTLV-4 have no associated diseases, which could be related to the different genomes, or to their cell tropism. However, the presented hypotheses have not yet been confirmed, so the scientists have no clue why these viruses are not pathogenic.

[1] Process described as the occurrence of mitotic divisions of HTLV-1 infected T-cells.

1.2. Pathology

HTLV-1 infection is chronic and asymptomatic for the vast majority of infected individuals. However, in 5-10% of cases, chronic infection is pathogenic with two aggressive diseases: Adult T-cell Leukaemia/lymphoma (ATLL) or HTLV-1 associated myelopathy/ tropical spastic paraparesis (HAM/TSP).

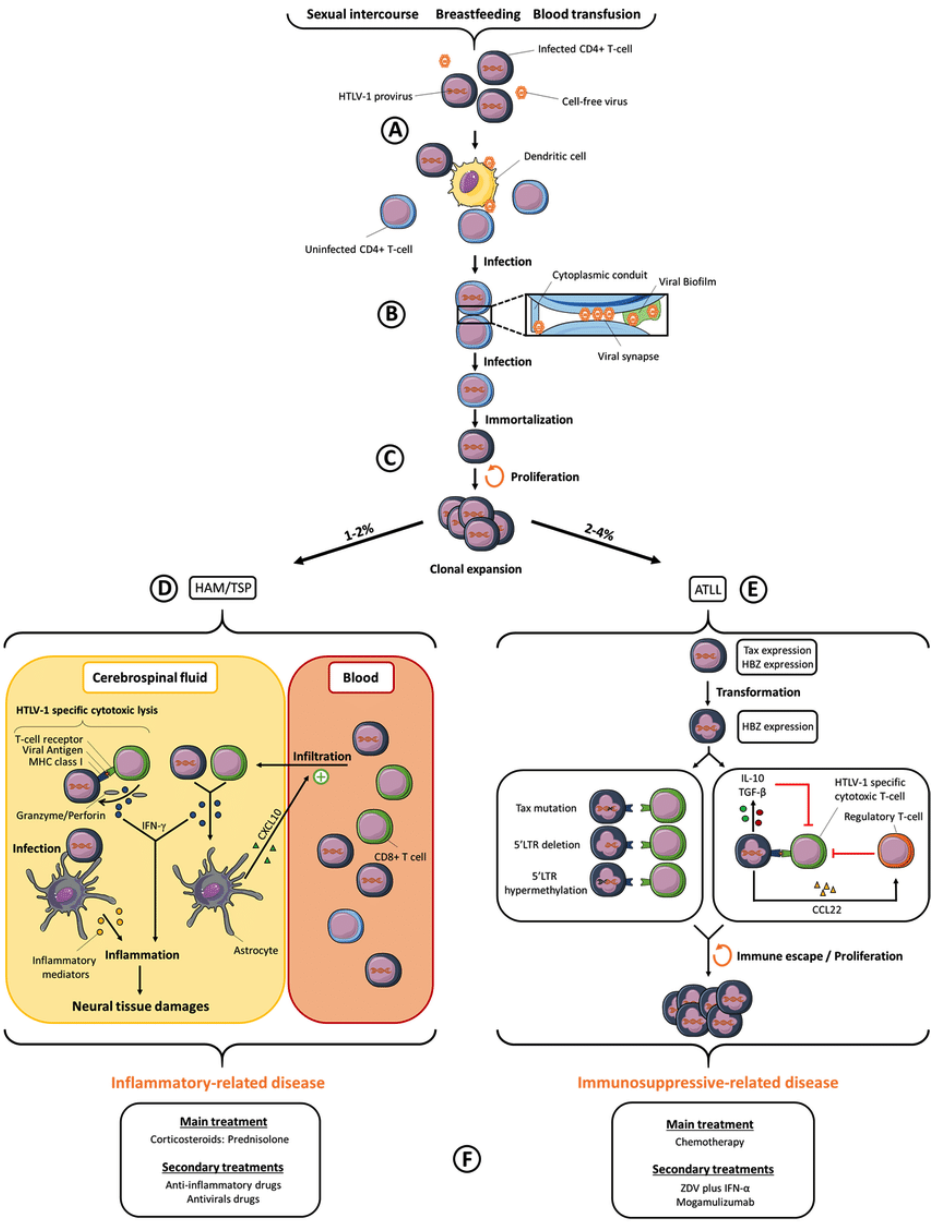


Figure 2 - HTLV-1 disease development. (A) Viral dissemination from infected cells to dendritic cells that are then able to transmit the virus to T-lymphocytes. **(B)** Infection of other T-cells after cell-to-cell contact (virological synapse or cellular conducts or viral biofilm) with infected T-cells. **(C)** Infected cells undergo cell signaling alterations, such as continuous proliferation through mitotic divisions and apoptosis inhibition. **(D)** TSP/HAM is an inflammatory disease caused by the IFN-y production by infected cells. INF-y is infiltrated in the cerebrospinal fluid, contributing to neural tissue damages. **(E)** ATLL is an immunosuppressed disease caused by the accumulation of genetic alteration in infected T-cells. These alterations allowed them to escape immune responses**. (F)** Options of treatment for TSP/HAM or ATLL patients**.** Image retrieved from Futsch *et al.* Viruses 2017. [8]

1.2.1. ATLL

Adult T-cell leukemia/lymphoma is characterized by unrestrained growth of T-cells that can be found in the blood, lymph nodes or multiple areas of the body. This disease causes both lymphotropic leukemias, in which the cancer cells are mainly hosted in the bone marrow (BM) and blood, and lymphomas characterized by the tendency of cancer cells to be mainly in the lymph nodes, yet they can be found in other tissues. For the chronic and less aggressive manifestations of ATLL, the life expectancy is estimated to be 30 to 55 months, and less than 10 months in the case of aggressive manifestations, such as the lymphoma, which represents more than 60% of all cases [9, 10].

The exact mechanism of ATLL pathogenesis is not fully elucidated. However, ATLL arises due to proliferation of HTLV-1 infected cells, with a progressive malignant transformation [11, 12]. This process is controlled, at least in part, by viral genes. Some regulatory proteins, encoded by the viral genes, as Tax and HBZ (HTLV-1 Basic Zipper Protein), have an important role in oncogenic process of ATLL, promoting viral persistence, growth stimulation and tumour development [12].

The protein Tax is indispensable for the activation of provirus[1] transcription. This protein can induce many types of cancer, depending on their promoters. Tax expression alters the cells' cycle by suppressing S-phase checkpoints and/or the DNA repair, which consequently induces genetic instabilities that are associated to oncogenesis. In approximately half of the cases, the ATLL cells lose Tax expression, due to punctual mutations on the *tax* gene, or due to hypermethylation's or delectations of the provirus 5'LTR (Long Terminal Repeat) (**Figure 2e**). On the other hand, HBZ is constitutively expressed in ATLL cells [8, 13-15]. This protein is more important in the clonal proliferation and survival of infected cells. HBZ protein promotes the upregulation of *Foxp3* gene, leading to the conversion of infected cells into Treg cells ($Foxp3^{+}CCR4^{+}$). Therefore, these cells become apt to hide HTLV-1 from the host's immune system and to transfer the virus to other individuals [15, 16]. In addition, infected cells produce anti-inflammatory cytokines, such as IL-10 (Interleukin-1) and TGF-β (Transforming Growth factor-beta), which promote the recruitment of more Tregs cells and the suppression of HTLV-1-speficic cytotoxic T-cells [8].

1.2.2. TSP/HAM

Tropical spastic paraparesis/HTLV-1 associated myelopathy is a syndrome in which HTLV-1 infection produces inflammation in the central nervous system, causing spastic weakness or paralysis of the legs, lower back pain and urinary dysfunctions [17]. Within the cerebrospinal fluid there are an accumulation of both CD4+ and HTLV-1-speficic cytotoxic T-cells (**Figure 2d**). These HTLV-1-infected cells secrete inflammatory cytokines, as IFN-γ (Interferon gamma), that contributes to the occurrence of neuronal damage, such as lesions

[1] Viral genome that can be integrated in the host DNA.

in glial cells, neurons, and astrocytes, as well as myelin loss. Moreover, IFN-γ also stimulates the production of CXCL10 (C-X-C motif chemokine ligand 10), a molecule that favours the infiltration of more HTLV-1 infected CD4+ cell into the inflamed tissues, establishing an inflammatory loop [8].

1.2.3. Other associated diseases

Other diseases are linked to HTLV-1 infection, and some studies show little evidence that HTLV-1 causes other forms of cancer. HTLV-1 infection can be linked with HTLV-1 associated uveitis (HAU), infective dermatitis, bronchiectasis, bronchitis, and bronchiolitis, asthma, ocular lesions, seborrheic dermatitis, Sjogren's syndrome, rheumatoid arthritis, ulcera colitis, fibromyalgia, chronic inflammation and accelerating ageing, such as cardiovascular and osteo diseases [18-22]. However, the mechanism of the virus driven pathologies are not clear.

1.2.4. Treatments

Chemotherapeutic treatments are the most used to treat ATLL patients, but they are not totally efficient, and they are frequently associated with secondary effects. Thus, new therapies aiming at targeting the virus have been developed. Some ATLL patients were efficiently treated with a combination of AZT (Zidovudine) and IFN-α (Interferon alpha) followed by chemotherapy. The use of AZT/IFN-α is the gold standard of treatments for ATLL leukemic patients; however, its use is not established in all countries [8, 23]. AZT/IFN-α treatment reduces HTLV-1 proviral load[1] and VEGF (vascular endothelium growth factor) levels in ATLL patients. VEGF has a crucial role in tumour survival thought inducing of angiogenesis and by increasing the resistant to chemotherapy for cancer [24]. Moreover, AZT/IFN-α induces p53 signalling and apoptosis of the HTLV-1 infected cells, *in vitro* [25]. Strikingly, most patients relapsed after short response to treatment suggesting that AZT/IFN-α alone is not sufficient for a sustained response or for a complete cure.

More studies have been performed to find antiviral therapies with high efficiency results. It has been demonstrated that, for example, IFN-β (Interferon beta) induces *in vitro* greater antiproliferative and proapoptotic effects than IFN-α, suggesting IFN-β as a strong candidate to clinical trials. Moreover, immunotherapy targeting CCR4 (CC receptor 4) is a different approach, that had promising antitumoral activity. It was reported that cancer cells from lymphoma patients are positive for CCR4. Therefore, this new therapy seems promising, and it is already in clinical trials [26].

Contrary to the therapies for ATLL, the remedies for TSP/HAM are designed to suppress the clinical symptoms, instead of eliminating HTLV-1 infected cells. These treatments resort to the use of corticosteroids, which usually present an effective anti-inflammatory response. However, recent studies have reported drugs that decrease HTLV-

[1] Marker of that quantify cell infection *in vivo*, increases either by cell proliferation or by viral replication.

1 proviral load and induce anti-HTLV-1 responses, *in vivo*. The combination of AZT and Lamivudine have been analysed in patients with TSP/HAM. Although, the results were not really sustained, they observed that there was a virological and clinical improvement and posterior decrease of HTLV-1 proviral load. This combination is already used in anti-HIV-1 therapies [27-29].

Despite all the research done in this subject, until now there is no treatment fully developed and available for both HTLV-1 associated diseases.

1.3. Motivation

HTLV-1 is a member of the *Retroviridae* family, such as the well-known HIV: the lentivirus associated with the acquired immunodeficiency syndrome (AIDS) [30]. This close similarity is illustrated by the story of HIV discovery, two years after that of HTLV-1, and described as a new virus associated with alterations of T-cell functions and numbers. In the first instance, and due to the similarities found between these two viruses, the scientists hypothesized that HIV was a third member of the HTLV family. However, soon they understood that it was a distinct retrovirus.
Although, these retroviruses are similar at both structure and transmission level, HIV-1 is much more infectious. In 2018, it was estimated that 37,9 million people are living with HIV-1 and that it was related to 770'000 deaths. In contrast, HTLV-1 infected 15 to 20 million people worldwide, and causes morbidity and mortality in only 5-10% of the infected individuals [4, 9]. While HTLV-1 has a long incubation time[1], around 20 to 30 years, HIV-1 infection will advance to AIDS, usually in 10 to 15 years if people do not receive the antiretroviral treatment [31].

Given the presented numbers of HTLV-1 prevalence and morbidity, it is easy to understand why this virus have been highly neglected and unknown by the general population and even by many health professionals. In addition, ignorance of this virus may be also related to either the lack of recognition of HTLV-1 associated diseases as a sexually transmitted disease, or to the geographical distribution and higher prevalence in specific populations, since it is mostly found in undeveloped countries [32].

Brazil is the country with the highest absolute number of positive HTLV-1 cases in the world, with around 700,000 to 2 million infected people [33]. Moreover, the values presented for the number of infected people in the world may be quite different from the real ones, since most of the population does not perform screening tests for HTLV-1 [34]. Normally, a person is diagnosed as seropositive for HTLV-1 only after an episode of ATLL or TSP/HAM manifestation or after screening of family member of ATLL or HAM/TSP patients. As HTLV-1 is transmitted horizontally through unprotected sex and vertically through prolonged breastfeeding, many women only discover their infection during prenatal examinations [35]. The form of transmission and clinical manifestations such as progressive motor disability, genitourinary disorders, as well as the restriction to breastfeeding, may have an impact on the daily lives of these people, generating social

[1] Time between the moment of infection and the developing of the symptoms.

discrimination and stigma. The social demand for breastfeeding leads to embarrassing situations for HTLV-positive women: besides the pain of not breastfeeding, mothers suffer society judgment. In addition, it has been reported that physical changes such as erectile dysfunction and overactive bladder in patients with TSP/HAM, promote reduced self-esteem, impair sexual interest, impede new relationships, and favors social isolation [33, 34].

Although the vast majority of those infected remain asymptomatic carriers throughout their lives, it is important not to neglect the many people suffering from ATLL or TSP/HAM: two highly debilitating and fatal diseases, nor the asymptomatic people who suffer on a personal or social level. The scientific study of HTLV-1 is therefore fundamental to design and develop methods of treatment and prevention, which until now are rare or non-existent. Specifically, the molecular mechanisms of how HTLV-1 is transferred from the infected cells to the target cells is not known but could lead to the identification of new therapeutic option aiming at blocking the viral transmission or the viral production.

Despite the numerous similarities between HIV-1 and HTLV-1, it is easy to identify that there is a major difference between these two human retroviruses malignancy, which could be justified by the differences on the active replication process. HTLV-1 lasts as cell-associated provirus, with low active replication. In contrast, HIV-1 exhibits high levels of active replication, which consequently leads to strong levels of detectable viremia. Moreover, while HIV-1 can spread by free particles, it has been reported that HTLV-1 virions are not infectious. On the other hand, it is well established that these two human retroviruses, can be transmitted from one host cell to another through cellular contact, a route that is far more efficient than the dissemination of free particles [36-38]. This mode of transmission is the only one that allows the HTLV-1 dissemination. Interestingly, infected cells produce and release free virions, while *in vitro* culture and even in that case, free HTLV particles are not infectious, which suggests that the virions produced for transmission by cellular contact might be different from those released in the medium. Two viral particle transfer pathways have been described for these two viruses: (i) virological synapse, (ii) the formation of intercellular conducts, and a third only for HTLV-1: (iii) the production of adhesive extracellular aggregates, or viral biofilms. These structures are mostly concentrated at one pole of the cell, and systematically found at the contact between cells during cellular transmission, and constitute an original mechanism of viral transmission, although still poorly characterized. Retention of retroviral particles on the surface of infected cells involves:

(i) remodeling of the extracellular matrix and its polarization.
(ii) specific addressing or assembly of virions at the level of these polarized extracellular structures.
(iii) physical interactions and physical resistance allowing the maintenance of virions and their integrity within the biofilms.
(iv) mobility and flexibility of the biofilms, to establish contact with the target cell and allow the transfer of virions to their target receptors.

During cell-to-cell contact, the biofilm rapidly adheres to the target cell allowing rapid transfer of HTLV-1. Since cell-to-cell contact is the main mode of HTLV-1 dissemination, in-depth

knowledge of this type of transmission and its molecular players is highly important, so that in the future it will be possible to develop a mechanism to prevent HTLV-1 transmission.

1.3. Objectives

In this project we propose to determine the composition of HTLV-1 biofilms and identify the components of these biofilms that are important for viral transmission. The human retroviruses, HTLV-1 and HIV-1, have the particularity of being transmitted very efficiently by contact between infected virus-producing cells and target cells. Due to the similarities between the two human retroviruses, HIV-1 and HTLV-1, many studies carried out on HIV-1 are based on results obtained for HTLV-1 and vice-versa. Hence, the choice of the proteins to be studied was related to the importance and the role of these proteins in the viral transmission of HIV-1 or with historical of these proteins in HTLV-1 transmission, as for example the SNAP25, which is a surface receptor involved in the virological synapse of HIV-1, and Caveolin-1, which is important for HIV-1 endocytosis and seems to be involved in the virologic synapse. Moreover, the host team demonstrated that these two proteins have their expression increased in infected cell lines and that there is a co-localization with the viral particles at the cell's surface. Hence, these two proteins represent then good candidates as component of HTLV-1 biofilm, potentially involved in its transmission.

Using different cell lines, the host team has also demonstrated that the composition and polarization of HTLV-1 biofilm appears to have a major impact on viral transmission and infection. **The objectives of my project were therefore to study the composition, and to identify the molecular mechanisms responsible for the transmission of HTLV-1 biofilms and the infection of target T lymphocytes.** To this end, we will try to silence the surface receptors, Caveolin-1 and SNAP25, and analyzed the density and composition of viral biofilm in the absence of these proteins. Moreover, we will study the importance of Caveolin-1 and SNAP25 for the viral transmission to target reporter cells, using imaging techniques combined with cell biology techniques. Then, we plan to extend this approach to study the influence of other proteins found in HTLV-1 biofilm to determine their role in HTLV-1 transmission. In addition, as HIV is also transfer by cell-cell contact and thought to be stored at the surface of infected cells, the long-term goal of our project is to use our results of the HTLV-1 transmission as a model to study HIV-1 viral transmission.

1.4. Contributions and partnerships

Coline Arone (PhD Student) and Dr. Delphine Muriaux, from IRIM Montpellier are working in partnership with Dr. Hélène Dutartre (my supervisor) for the project of characterization of the viral biofilm.

Chapter 2

Background

2.1. Structure and genomic organization

HTLV-1 is a round-shaped, enveloped virus of approximately 80-100 nm in diameter. The outer layer of a HTLV-1 virions consists of a membrane-associated matrix protein and a lipid layer intersected by the envelop proteins **(Figure 3b)**. Beneath this structure, the icosahedral capsid enables protection of the single-stranded diploid RNA genome which is complexed with the viral enzymes: reverse transcriptase, integrase and protease [30].

The viral genome of HTLV-1 is 9030-9040 nucleotides long and contains two flanking long terminal repeat (LTR) sequences. This virus has a more complex transcriptional processing than the simple retroviruses [30, 39]. Indeed, the genome encodes for many non-structural proteins besides the structural proteins **(Figure 3a)** found in all retroviruses [39].

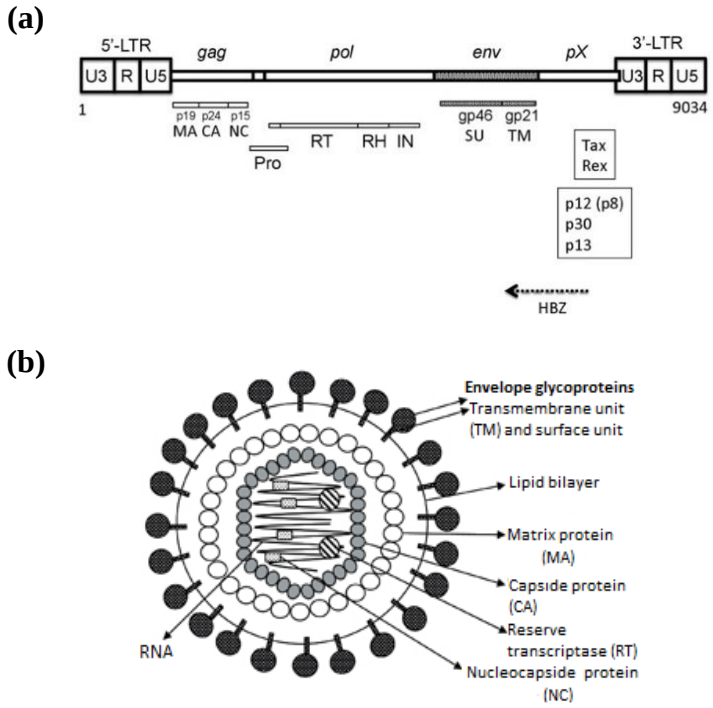


Figure 3 - HTLV-1 genome and structure. (a) Genome of HTLV-1: the matrix (MA), capsid (CA) and nucleocapsid (NC) proteins are encoded by the gag gene. The env gene encodes for two proteins important for the fusion and entry of the virus into the host cells. The pol gene encodes reverse transcriptase (RT), RNase H (RH), and integrase (IN). The p13, p12, p8, p30 proteins are encoded by the pX region. HBZ is encoded by the anti-sense frame of the provirus [39]. **(b) Schematic structure of HTLV-1 virion**. Image modified from Gillet, N. *et al.,* 2007 [40].

First, the ***gag* gene** is encoding the Gag polyprotein which will lead to three viral domains (matrix (p19 MA), capsid (p24 CA) and nucleocapsid (p15 NC)). When the viral protease processes the Gag protein, the three domains become individual viral proteins with specific functions. The structural Gag protein is the most important protein in the retrovirus particle [39, 41].

The matrix domain (p19 MA) is located at the N-terminal of the Gag polyprotein and contains the residues responsible for the recruitment of Gag to the cell membrane. The structurally conserved capsid domain (p24 CA) in its CA amino-terminal domain (NTD) has a centralized coiled-coil-like structure of six alfa-helices, and four alfa-helices in the CA carboxy-terminal domain (CTD).

The CA-CA interaction drives the Gag oligomerization and Gag-Gag interaction during virus assembly. This process controls both the viral particle morphology and size, plus the formation of the core during virus maturation [41]. Finally, the NC domain of the Gag polyprotein possesses two zinc finger domains that are important for the Gag-membrane binding. Thus, the nucleocapsid encapsulates the viral genomic RNA, due to its packaging signal [41].

The ***pol* gene** encodes for three enzymes: reverse transcriptase (RT), RNase H (RH) and integrase (IN). The viral protease (Pro) is encoded by the ***pro* gene**. In many retroviruses, like Rous sarcoma virus (RSV) and human immunodeficiency virus, the Gag or Gag-Pol polyprotein precursors are encoded by the *gag* gene. However, HTLV-1 has two distinct ribosomal reading frames: one from both *gag* and *pol* and a second one for the *pro* gene. In another words, the same nucleotide sequence can be divided into a set of consecutive, non-overlapping triplets and the ribosome may shift from one frame to another during translation of the viral RNA [42].

The protein synthesis from the ***env* gene** gives rises to the surface unit (SU) which is a 62-kDa protein (gp62). Then the SU is cleaved into a SU gp46 and a transmembrane unit (TM) gp21 proteins.

Finally, the **pX region** encodes for the Tax and Rex proteins plus for accessory proteins as p12 (p8), p30 and p13. The REX and TAX are very important in the HTLV-1 cycle. They are coded in open reading frames (ORFs) [43]. The anti-sense frame corresponding to the pX and *env* gene region of the provirus encodes for the HBZ protein [30, 39].

2.2. Viral cycle

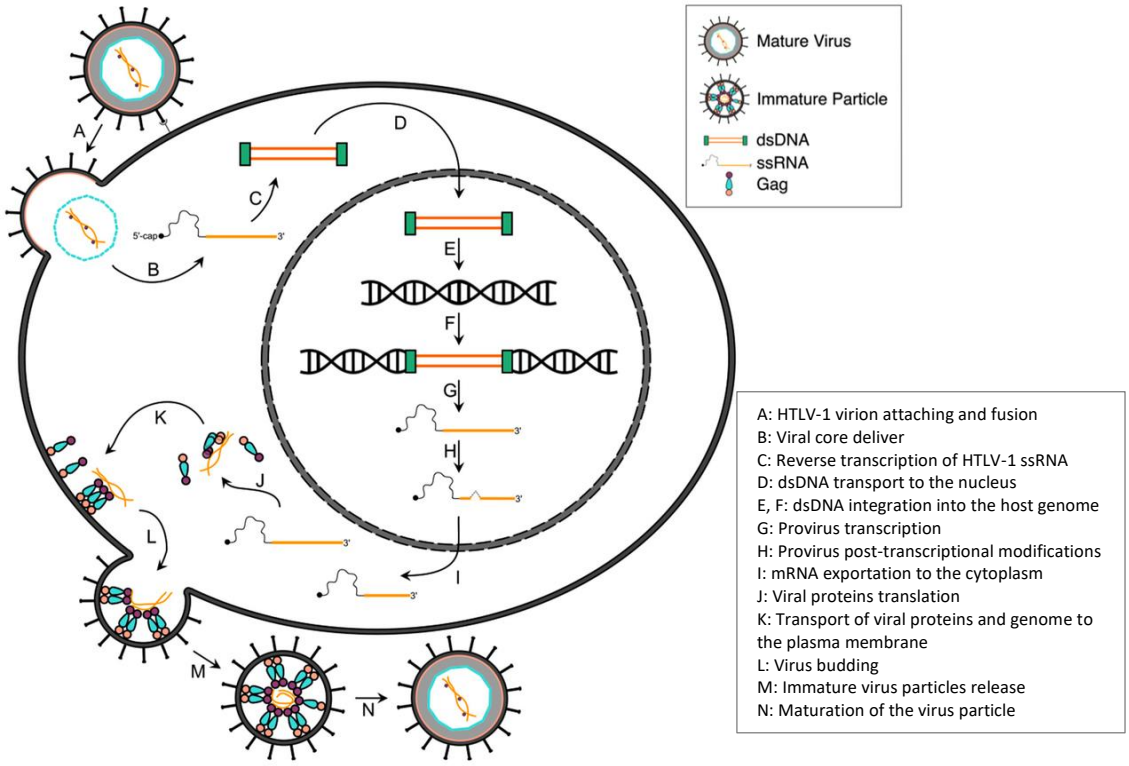


Figure 4 - HTLV-1 life cycle. Schematic representation of the major steps in the life cycle of HTLV-1 within CD4+ cells. Image retrieved from Martin *et al.* Viruses 2016 [44].

2.2.1. Fusion and entry

HTLV-1 has tropism for many cell types, including $CD4^+$ and $CD8^+$ T-cells, B cells, macrophages, myeloid cells, and fibroblasts due to the extensively distributed cellular surface receptors: GLUT-1 (glucose-transporter 1), HSPG (heparan sulphate proteoglycan) and NRP-1 (neuropilin receptor 1) [45]. During the early binding point, HTLV-1 SU gp46 interacts with the HSPG (core proteins associated with one or two of the sulphated polysaccharide side chains heparin sulphate (HS) glycosaminoglycan). This binding would enhance the stability and interaction of the gp46 with NRP-1, (a 130kDa single membrane-spanning glycoprotein) forming a HSPG/NRP-1/SU complex [39]. Following this event, GLUT-1 (a 12-membrane-spanning receptor for glucose transport across the cell membrane) associates with the complex through the binding of the NRP-1 and the GLUT-1 C-terminal binding protein [46]. Then, a conformational change occurs and allows the communication of the gp46 SU with GLUT-1, promoting the gp21 TM translocation and fusion **(Figure 4a)** [45].

Independently of the HTLV-1 infection, GLUT-1 is found in cell contact sites. However, NRP-1 is concentrated in membrane junctions formed between HTLV-1 infected and uninfected T-cells and partially co-localized with GLUT-1 [46]. This suggests that NRP-1 expression and recruitment are supported by a specific, but unknown mechanism present in transformed cell lines used *in vitro* and in the main target cells of HTLV-1 infection *in vivo*.

2.2.2. Reserve transcription, nuclear transport, and integration

After HTLV-1 fusion with the target cell membrane, the viral capsid core containing reverse transcriptase, protease, integrase and the two copies of viral genomic RNA (vRNA) is released into the cell cytoplasm **(Figure 4b)** [47]. However, complementary studies have yet to be done to clarify the correlation between HTVL-1 CA uncoating and reverse transcription occurring after viral entry. Although it has not been widely studied, due to its difficulty to be produced *in vitro* for enzymatic studies, the reverse transcriptase allows the synthesis of double-standed DNA (dsDNA) from HTLV-1 RNA **(Figure 4c)**. Reverse transcriptase is a 95kDa enzyme with DNA polymerase and RNAse H activity [48]. The HTLV-1 RT could be a p62/p49 heterodimer, analogous to the p66/p51 RT heterodimer of HIV-1. The HTLV-1 p49 protein cannot perform reverse transcription by itself. RT activity requires the catalytic p62 subunit [42].

After reverse transcription, the new viral dsDNA is integrated into the host cell chromosome by the viral integrase and lead to the provirus form **(Figure 4d/e/f)**. The nuclear import still needs to be elucidated. However, it is thought that viral integration occurs during mitosis of infected T-cells, in which the nuclear membrane has been dislocated [49].

As detailed below, HTLV-1 can infect *in vitro* T-cells, but also DCs, which raises many questions regarding how reverse transcription, nuclear transport, and viral DNA integration are performed in DCs [36, 50, 51]. Specifically, it remains unclear how RT can proceed in presence of SAM domain and HD domain-containing protein 1 (SAMHD1) a

restriction factor known to lower the pool of nucleoside and thus described as inhibiting HIV-1 RT. Interestingly, HTLV-1 was reported to be insensitive to SAMHD1 restriction, but the mechanism of this escape is not known [52]. More importantly, after its reverse transcription, how the viral genome is transported to the nucleus of DCs is still puzzling, because these cells are non-mitotic, and thus their nuclear membrane remains intact throughout their life [53]. Finally, it is still not known whether integration sites are similar in mitotic T-cells and non-mitotic DCs.

2.2.3. Gene transcription and post-transcription regulation

The LTR of the HTLV-1 provirus bares a promoter and enhancer that are essential to initiate RNA transcription **(Figure 4g)** [54]. This viral genome is flanked by 2 LTRs that both are transcriptionally active leading to sense- and antisense-transcription. The sense orientation of the LTR (5'-LTR) have a promoter with a DNA region called TATA-box. In contrast, the promoter in the antisense-orientation (3'-LTR) does not have this TATA-box structure, thus harbouring lower transcriptional plasticity. HTLV-1 replication cycle is controlled by two proteins TAX and REX [43].

The TAX protein is a robust positive regulator of the sense transcription from the 5'-LTR during the early phase of infection [55]. There are three conserved 21-bp repeat elements called Tax-response element (TRE) that are receptive to the transactivation mediated by the viral protein Tax. The TRE binds to the cyclic AMP response element binding protein (CREB) through its N-terminus (NTD), while the C-terminal domain (CTD) of Tax promotes the transcriptional initiation by interaction with the TATA binding protein [56]. The multifunctional cellular co-activators CREB binding protein (CBP), p300 and p300/CBP-associated factor are recruited by the Tax-CREB complex to the LTR [57].

Many other factors can affect the regulation of transcription. The HTLV-1 p30 nuclear protein has the ability to inhibit the interaction between Tax and p300, resulting in the suppression of the 5'-LTR transcription. The HTLV-1 p13 is a protein with anti-proliferative activity, which can be found in the inner part of the mitochondria's membrane. HTLV-1 p13 can also become ubiquinated in the presence of Tax and be translocated to the nucleus where it promotes an inhibitory effect on the physical interaction between Tax and p300. Furthermore, HBZ competes with Tax for the CREB protein and p300 binding, so HBZ suppresses the 5'-LTR transcription [55, 58]. Finally, the 3'-LRT promotes the expression of HBZ which can be controlled by Sp-1 and Jun-D [59].

The HTLV-1 provirus can also have an epigenetic regulation, which is not static nor predictable, because it depends on the intra-cellular and/or extracellular conditions [55]. HTLV-1 LTRs contain CpG islands which are present in gene promoter regions of the host cells. The DNA methylation of CpG islands can affect the nucleosomal positioning and promote gene silencing [60]. Another example are histone modifications like acetylation, phosphorylation, methylation and ubiquitylation [61].

The nucleosome is the fundamental unit of chromatin, which is composed of eight histones. It is known that histone acetylation contributes to a strong sense transcription from 5'-LTR, and the complex Tax-CREB is able to promote the acetylation of histones [61]. Whether a similar transcriptional activation and regulation occurs also in DCs is not yet investigated.

Recent studies also showed that physiological hypoxia (1% or 2% oxygen), can enhance HTLV-1 transcription. In the organism it is possible to find these low levels of oxygen in the lymphoid organs and bone marrow. This new idea can be related with the HTLV-1 transmission to HSCs and pDCs, as already described, due to their localization [62].

After the transcription there are some cellular factors or events able to regulate the splicing and the transport of the viral mRNA. When Rex accumulates in the nucleus, it reduces splicing and regulates the transport of unspliced (*gag-pro-pol*) and singly spliced (env) HTLV-1 mRNA. Rex also is responsible for the transport of the doubly sliced (*tax, rex, p30II, p12, p13 and hbz*) viral mRNA by interaction with the Rex-responsive elements (RexRE) which are the U3 an R sites of HTLV-1 RNA in 3'-LTR [63, 64].

Rex also has a nuclear export signal, at the activation domain, allowing the targeting and movement between the nucleus and cytoplasm [65].

2.2.4. Particles assembly, building, maturation, and exportation

The late phase of the HTLV-1 life cycle is the assembly of viral RNA and proteins, which result in the release of virions. The Gag polyprotein is the key structural protein in this process.

The HTLV-1 assembly pathway requires genome recognition (Gag-RNA interaction) as well as Gag-Gag and Gag-cellular protein interactions [66].

Very little is known on how the virus assembles. Most of the knowledge is based on HIV studies. However, there are some processes that are different and need to be well understood. The trafficking of the Gag polyprotein (Gag-Pol) to the plasma membrane is the primary step to the assembly **(Figure 5)** [66, 67]. The beginning of the particle assembly can occur in the cytosol, but it is apparent only when the RNA is already at the cell membrane. The Gag NC domain interacts with high affinity to the HTLV-1 RNA, and it is the most important domain for viral RNA packaging into the particles. In addition, the Gag MA domain seems also plays a role in specific interaction with the viral RNA. Moreover, the Gag CA domain is involved in Gag-Gag interactions and Gag-protein interactions, via CA-CA binding [66, 68].

Where these Gag-Gag interactions take place is also a matter of debate. Using fluorescence fluctuation spectroscopy (FFS) to quantify the interactions of fluorescently labelled proteins it was demonstrated that the concentration of cytoplasmic HTLV-1 Gag is very low compared to that of HIV-1 Gag protein. This supports the idea that HTLV-1 reaches the cell membrane as a monomer, in contrast to HIV-1 [66, 67]. However, the

detection of oligomers can be challenging due to the low expression levels of HTLV-1 Gag and using cellular spatial resolution [67, 69-71]. Nowadays it is known that both viruses have interactions at the cytoplasm before reaching the PM and that the real difference is that HIV-1 requires micromolar concentration of Gag to be able to associate with the PM, while HTLV-1 just requires nanomolar concentrations [66, 67]. Based on that, it is understandable that at the beginning scientists assumed an absence of HTLV-1 Gag interaction in the cytoplasm. Interestingly, in DCs HTLV-1 seems retained in vesicles suggesting that viral assembly may use a different process than in T-cells.

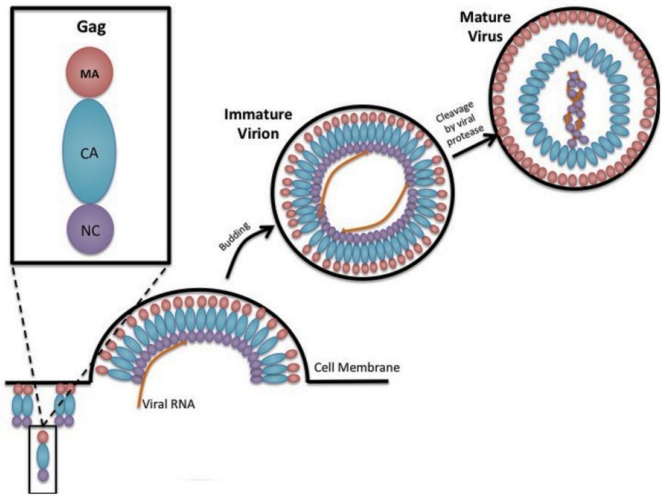


Figure 5 - Hypothesis for Gag and retrovirus particle assembly. Gag oligomerization along the inner side of the plasma membrane is observed during the assembly of a prototypic retrovirus particle. The Gag protein is composed of a nucleocapsid domain (purple circle), a capsid domain (blue oval), and a matrix domain (red circle). Two copies of the viral RNA (orange lines inside the viral particle) are shown packaged into the virus particle. Image retrieved from Maldonado et al. Frontiers in Microbiology, 2014 [67].

Finally, for the interaction with the PM, the MA domain of Gag contains a bipartite membrane binding signal, which can interact with negatively charged lipids due to its positively charged amino acids, and that allows the identification of virus budding sites and the insertion into the membrane. Cellular factors are also recruited to these sites, resulting in budding (Gag-cellular proteins interactions) and subsequent release of immature particles [66]. After, the viral protease cleaves the Gag and Pol, CA forms the capsid shell that contains integrase, reverse transcriptase and gRNA linked to the nucleocapside and MA remains associated with the plasma membrane until viral particle release [72]. The conversion of immature virus particles to mature infectious particles is marked by protease catalysis.

2.3. Tropism

2.3.1. T Lymphocytes

HTLV-1 can infect many cell types by the binding of the viral envelop proteins to the HTLV-1 receptors at the target cell surface.

HSPGs, GLUT-1 and NRP-1 are receptors involved in the binding and entry of viral particles into the target cells [73]. These receptors can be found both at the surface of CD4+ and CD8+ T-cells, as well as at the surface of almost all cell types. Thus, one could expect that most, if not, all cells would be infected by HTLV-1. The HTLV-1 proviral load, that is used as a marker of cell infection *in vivo*, increases either by cell proliferation (clonal expansion) or *de novo* infection (viral replication). These two mechanisms influence the number of infected cells found *in vivo* and may also be different in diverse cell types. The cell population with the highest viral load is used to characterize the viral tropism [74].

In vitro studies demonstrated that HTLV-1 infects both CD8+ and CD4+ T-cells; this is reliable with the fact that the viral surface receptors are similar. I*n vivo,* the tropism is different, with mainly CD4+ T-cells infection [73]. Since both CD4 and CD8 T-cells can proliferate using clonal expansion independently from their infection, it is plausible to suppose that HTLV-1 preferential tropism in CD4 T-cells might be related to the receptor distribution or concentration, rather than differential expansion of these T-cells.

Examination of cell surface markers revealed that CD8+ T lymphocytes expressed significantly lower levels of HSPGs than activated CD4+ T-cells. On the other hand, the latter cells express lower levels of GLUT-1 than CD8+ T-cells [73]. These studies imply that receptor abundance might not drive the tropism observed *in vivo*, except if HSPG abundance is more important than GLUT-1 abundance for the HTLV-1 infection. Hence, the main tropism of HTLV-1 for CD4+ T-cells can be associated to the expression levels of those receptors, but the surface receptor concentration is not the only variable to take into account. Indeed, the sequential binding to the different receptors might explain the differential tropism.

Studies done by *Jones K. S. et al*, and performed with a full-length HTLV SU, showed that HTLV-1 can bind first to HSPG, instead of GLUT-1 [75, 76]. Which came to contradict the previous studies on HTLV-1 binding. Moreover, membrane fusion leading to productive infection is the result of sequential HTLV-1 binding events to the receptor proteins at the target cell [46], and it is the result of interaction with GLUT-1 that allows fusion.

Alternatively, *Melamed A. et al* showed that HTLV tropism might also be related to the genomic site of integration and to the transcriptional orientation of the provirus [6, 73]. To sum-up, HTLV-1 tropism cannot be reduced to the presence and abundance of its receptor at the surface of cell harbouring the PLV.

2.3.2. Other cell types

Although HTLV-1 have mainly tropism for T-lymphocytes *in vivo*, viral DNA was found in myeloid cells such as monocytes or macrophages, dendritic cells, plasmacytoid dendritic cells (pDC), epithelial and endothelial cells [77], suggesting that HTLV-1 can also infects these type of cells. The infection of pDC is very intriguing, because these cells are major type I interferons producing cells in response to the viral infection, especially through sensing of viral RNA by toll-like receptor 7 (TLR7) [78]. Therefore, plasmacytoid dendritic cells are identified as resistant to any viral infection. Thus, the presence of HTLV-1 DNA in pDCs was intriguing for many years and subjected to controversies [79].

Recently, *Futura, R. et al* detected viral integration in hematopoietic stem cells (HSCs) and in other myeloid differentiated cells. Thus, it is possible to suppose that pDCs have HTLV-1 genome due to their progeny. Because HSCs have the ability to give rise to all myeloid cell lineages including pDCs, myeloid dendritic cell and monocytes, one can hypothesized that pDCs have inherited HTLV-1 genome as result of differentiation of infected HSCs into pDCs, rather than productive infection of the already differentiated pDCs, which, furthermore could not be reproduced *in vitro* [80]. Moreover, similar results were found for monocytes. These cells have PVL *in vivo*, but when researchers tried to infect them *in vitro*, the cells could not support the infection and consequently they died [52, 81]. Hence, if we suppose that the infection if before the differentiation of the HSCs into monocytes, we can justify the results obtained.

How the HSCs become infected remain to be understood. However, there are two reasonable hypothesis in study: (1) in parallel to what happen during infection with HIV-1, CD4+ T-cells infected by HTLV-1, can circulate in the blood and then migrate to the BM, where they can infect HSCs or cells from their lineage; (2) infected DCs from the mucosal can migrate to the peripheral blood and, after that, do homing to the BM, promoting the infection of the local cell types [80, 82]

It has been reported that HAM patients show high levels of Tax-expressing cells in their BM [83]. As Tax is indispensable for viral replication and transmission, this suggest that there might be *de novo* infection of HSCs *in vivo*, supporting the hypothesis of viral transmission to their lineage cells. HSC infection and viral transmission to their lineage can explain some HTLV-1 tropism, but nowadays how HSC get infected is still unknown.

2.4. Summary

HTLV-1 is an oncogenic retrovirus leading to lifelong chronic infection. Some regulatory proteins, encoded by the viral genes, as Tax and HBZ have an important role in oncogenic process of ATLL, promoting viral persistence, growth stimulation and tumour development. The protein Tax is essential for the activation of provirus transcription and consequently *de novo* infection. On the other hand, HBZ is more important in the clonal proliferation and survival of infected cells.
Chronic infection is also associated with two aggressive diseases, ATLL and TSP/HAM. ATLL is characterized by the proliferation of HTLV-1 infected T-cells, with a progressive malignant transformation that causes both lymphotropic leukemias and lymphomas. In contrast, TSP/HAM is a syndrome in which the HTLV-1 produces inflammation in the central nervous system, causing spastic weakness or paralysis of the legs and urinary dysfunctions.

HTLV-1 infection occurs usually through breastfeeding and sexual contact. HTLV-1 infects many cell types. Although, *in vitro* studies demonstrated that HTLV-1 infects both CD8+ and CD4+ T-cells, *in vivo,* HTLV-1 is mainly found in CD4+ T-cells.

The infectious cycle of HTLV-1 begins with its binding to several receptors of target cells followed by entry of the virus. During the early binding steps, HTLV-1 SU gp46 interacts with HSPG and then with NRP, forming a HSPG/NRP-1/SU complex. Following this event, GLUT-1 associates with the complex promoting the gp21 TM translocation and the viral fusion with the target cell membrane. Once inside the cell's cytoplasm, the reverse transcriptase, protease, integrase and the two copies of viral genomic RNA are released. Then, reverse transcription occurs forming dsDNA which will be integrated into the host cell's genome. The transcription of the genes that encode for the viral proteins takes place within the nucleus. The respective mRNAs are exported to the cytoplasm and viral proteins are produced by translation. The late phase of the HTLV-1 life cycle is the assembly of viral RNA and proteins, which results in the release of immature virions that will be converter to mature infectious particles after protease catalysis.

Chapter 3

State of Art

3.1. HTLV-1 transmission

The natural HTLV-1 infection occurs through prolongated breastfeeding and sexual contact, and nosocomial infection through contaminated blood or by transplantation of infected organs and tissues [4]. The sexual transmission happens more efficiently from men to women than the other way around. The existence of other sexually transmitted diseases that can damage the mucosa, like Syphilis or Herpes, facilitates HTLV-1 transmission [77]. During natural infection, infected cells from the donor need to cross the mucosa of the digestive or sexual tract, before being able to transmit the virus to host cells, which usually are T-lymphocytes or DCs [70]. T-cells are not abundant at the mucosa, on the other hand, there are many DCs located in all mucosae, suggesting that they might be the first cells encountered by HTLV-1.

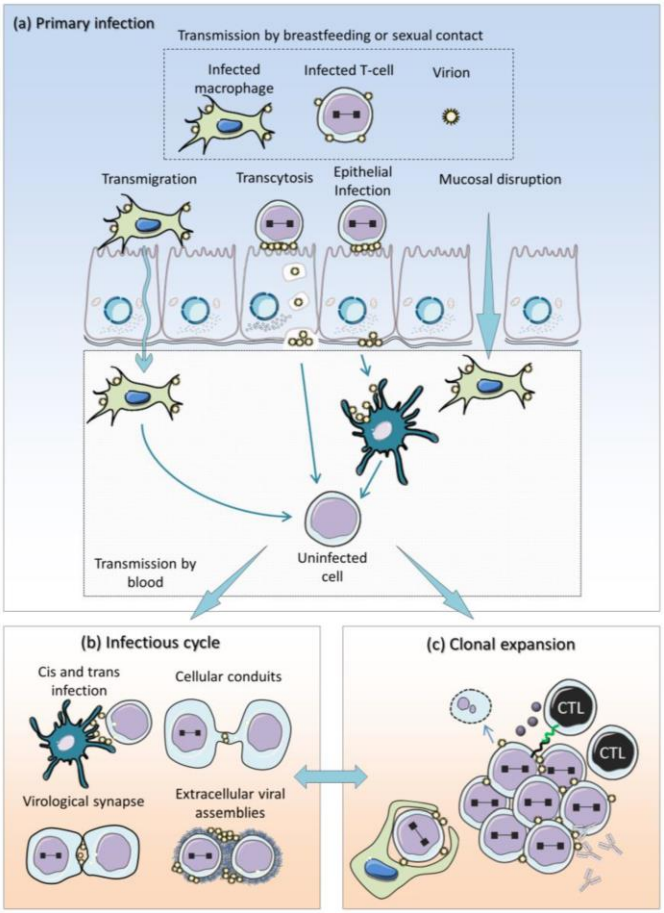


Figure 6 - Model of HTLV-1 replication *in vivo*. (a) Viral transmission by breastfeeding or by sexual contact. **(b)** Cell-to-cell transmission of the HTLV-1 virions, by three different routes. **(c)** HTLV-1 clonal expansion guide by mitotic division of infected cells. Image retrieved from Carpentier *et al.* Viruses 2015 [*84*].

The HTLV-1 can pass through the mucosal barrier by four mechanisms **(Figure 6)** [7]:

(1) transmigration of HTLV-1-infected macrophages.

(2) transcytosis of viral particles produced by HTLV-1 infected cells.

(3) release of newly produced virions from the basal surface of an infected epithelial cell.

(4) bypass of HTLV-1 infected cells through a disrupted mucosa [85].

Moreover, DCs at the mucosa can be infected and might then be able to transmit the virus to the lymphocytes [7]. Alais S. *et al* showed that DCs are more susceptible than autologous T-cells to HTLV-1 infection [36]. DCs can transfer HTLV-1 virions to T-cells by, at least, two mechanisms. First, DCs can capture viral particles either near to the mucosa or at the secondary lymphoid organs and then transfer them to a target cell (*trans-infection*). Or the other hypothesis is that, after DCs infection, they can produce newly made virions and infect other cells (*cis-infection*) [77]. The infection of other cell types by DCs, is possible since DCs are known to interact often and closely with T-cells, for example during antigen presentation. Thus, it is plausible to think that DCs are linked with T-cells infection, and that they play an important role in establishing chronic infection. However, it is not clear when and how it happens. After the *de novo* infection by HTLV-1, there is a clonal proliferation of the infected cells, which is crucial to maintain the steady state of infection, as well as the number of infected cells. The clonal expansion happens when these new infected T-cells proliferate by mitotic divisions allowing *per se* the amplification of their provirus. This process maintains a steady state of infected cells and is controlled by viral proteins, such as Tax [85].

3.2. HTLV-1 transmission and infectivity *in vitro*

In 2008, *Jones et al.* suggested that cell-free viruses are poorly efficient at infecting T-cells, yet they could easily infect DCs. Jones and her team incubated DCs and monocytes derived dendritic cells (MDDCs) with cell-free HTLV-1. To distinguish the newly made particles from those internalized, the cells were also stained for Tax protein. This protein is absent from the viral particles, and only expressed in target cells after their *de novo* infection. At later times, the results indicated that DCs were expressing Tax and viruses were also detected in the culture's supernatants [50]. These results suggest that DCs can be infected by cell-free virus and consequently that they can produce new virions. Thus, it was proposed that DCs might be more susceptible for HTLV-1 cell-free infection than T-lymphocytes.

Then after, *Alais, S. et al.* demonstrated that newly made virions are not infectious neither to T-cell nor to DCs. *Alais, S.* and her colleagues promoted the infection of MDDCs and autologous T-cells using cell-free viral particles present in the supernatant of infected cells, highly concentrated viral particles solution and cell-free viruses embedded in a biofilm **(Figure 9)**. They demonstrate that the virions present in the biofilm were most efficient form of infection. Both supernatant containing cell-free forms and very concentrated supernatant are inefficient at infecting either T-cells or autologous MDDCs [36]. These contradictory results can be related to the cell-free form used by *Jones KS et al.* Indeed, in their experiment, cell-free particles were obtained after long term culture of highly concentrated cultures and were not demonstrated to be free of biofilm-like structures. Given that, viral biofilm can be released from the surface of infected cells after gentle agitation, it is likely that supernatants used by *Jones et al* could contain cell-free viruses associated to viral biofilms, which would thus justify their results [36, 50].

In conclusion, cell-cell contact, either in T lymphocytes or DCs, is the most efficient route of transmission. This is a unique characteristic of HTLV-1 as other retroviruses can efficiently spread using both routes [36].

Nowadays, it is assumed that cell-to-cell HTLV-1 transmission occurs via cellular conduits, via biofilm transfer or via the formation of a viral synapse [77].

3.2.1. Cellular conduits

The HTLV-1 conduits are nanotubules formed from the polymerization of actin-F. These structures generate protuberances at the plasma membrane of an infected T-cell that elongate towards an uninfected cell, allowing cell-to-cell communication and the exchange of material, such as proteins and viruses. As described before, HTLV-1 transmission is highly dependent on cell-to-cell contact, as cell-free virions are poorly infectious. In culture, many T-cell lines proliferate as aggregates, so viral transmission is favoured in close cell-cell communication, rather than long-distance communication. Indeed, both virological synapse and viral biofilm are believed to be more common as routes of HTLV-1 transmission [86]. Yet, it has already been shown that cellular conduits may be involved in the stabilization of the virological synapse. These actin nanotubules allow the transmission of the viral p8 protein, which promotes an increase in the number and size of conduits. Furthermore, the p8 protein promotes the expression of LFA-1 (lymphocyte function-associated antigen) by the non-infected cells, which consequently increases the number of interactions with the ICAM-1 (intercellular adhesion molecule) present at the surface of a target cell. This interaction is also a feature present during virological synapse formation [87]. Therefore, viral conduits can be involved in both viral transmission and viral synapse stabilization.

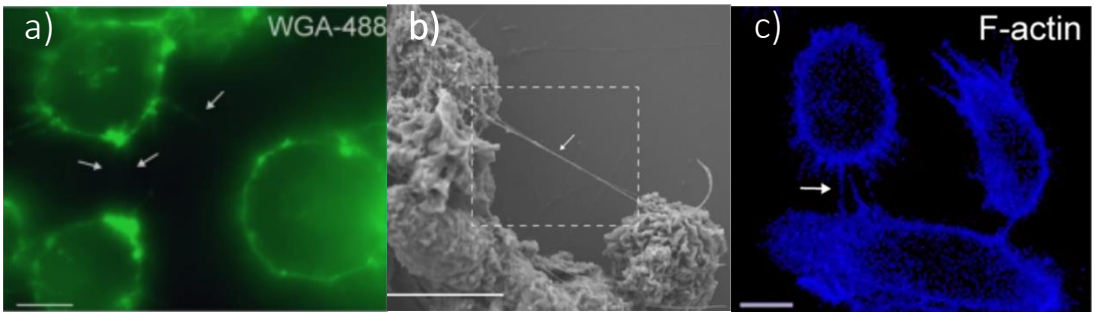


Figure 7 - HTLV-1 positive cells forming cellular conduits. (a) MT-1 cells stained with WGA-488 (green) and analysed by fluorescence microscopy. Arrows indicate the intercellular structures of connection. **(b)** Scanning microscopy (SEM) of MT-2 cells connected by a cellular conduit. The arrow indicates the intercellular structure of connection. **(c)** MT-2 cells stained with F-actin phalloidin-AF350 (blue) and analysed by confocal microscopy. The arrow indicates the structure of connection intercellular. Imagens retrieved from Omsland *et al.* Scientific Reports 2018 [*86*].

3.2.2. Virological Synapse

The virological synapse (VS) **(Figure 8)** is defined as a "virus-induced, specialized area of cell-cell contact that promotes the direct transmission of the virus between cells" [77].

This structure has been visualized and analysed in T-cells, but not yet in DCs. In 2003, Igakura *et al.* demonstrated that isolated HTLV-1 infected T-cells have an unpolarized distribution of the viral Gag and Env proteins, since these proteins appear to be distributed randomly around the cell's periphery **(Figure 8a, b)**. However, the same study showed that during cell contract, the viral proteins are polarized toward the contact's region formed with the target cell **(Figure 8c)**. The virologic synapse is then associated to an accumulation of viral genome, Gag and Env proteins at the junction space between the two intervenient cells (cleft synaptic).

The contact between an infected lymphocyte and an uninfected T-cell is promoted by the interaction of ICAM-1 on the HTLV-1 infected T-cells with the LFA-1 on the target cell. At the virologic synapse there is also an interaction of Env with its receptors, and signals induced by the viral Tax. This process causes the polarization of the microtubule organization center (MTOC) and the reorganization of the cytoskeleton inside the infected cell towards the target cell, allowing cell-cell adhesion. The polarized secretion of Gag p19 and Env proteins with the accumulation of viral genome at the interface between both cells allows viral budding inside the viral synapse cleft. The interaction between ICAM-1 and LFA-1 forms an outer ring that surrounds the viral proteins and genome, protecting them from the recognition by the immune system [77, 88, 89].

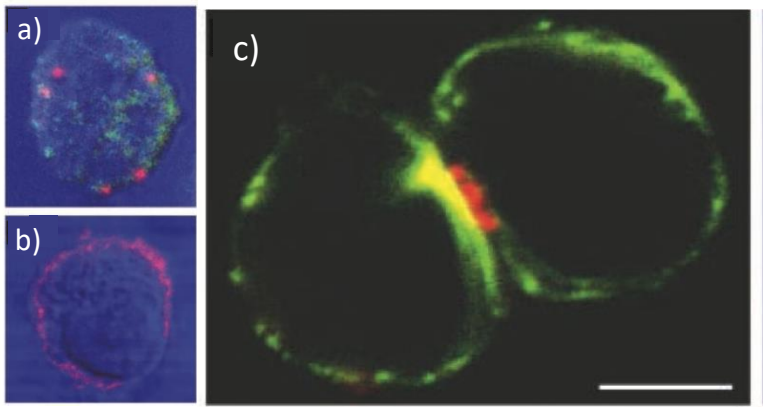


Figure 8 - Viral synapse (VS). (a) Distribution of tubulin-alpha (green) and Gag p15 (red) in an isolated T-cell analysed by confocal microscopy. **(b)** Distribution of Env pg46 protein (red) in an isolated T-cell analysed by confocal microscopy. **(c)** Distribution of tubulin-alpha (green) and HTLV-1 Gag p19 (red) during T-cells contact, analysed by confocal microscopy. Images retrieved from Igakura *et al.* SCIENCE 2003, [89].

3.2.3. Viral Biofilm

The ECM (extracellular matrix) is a three-dimensional non-cellular network composed of collagen, proteo- and glycosaminoglycans, elastin, fibronectin, laminin, and several other proteins and cellular receptors. This structure provides a physical scaffolding for the cellular components, and it is crucial for the morphogenesis, cell adhesion and migration, intercellular communication, and even differentiation [90, 91]. Apart from eukaryotic cells, bacteria and fungi can also be embedded in an ECM [92]. Bacterial biofilms are known as complex and dynamic communities, in which bacteria attach to host natural or artificial

surfaces and produce extracellular polymers that will facilitate the attachment and formation of the ECM. This structure is composed of polysaccharides, proteins, and extracellular DNA. Bacteria within a biofilm are metabolically and morphologically very different from the free-floating ones. They have protection from harmful conditions in the host, like the immune responses, nutrient deprivation, pH changes, disinfectants and even antibiotics. The biofilm is also resistant to the shear forces produced by blood flow [91, 93].

In 2010, Pais-Correia *et al.* showed that HTLV-1 particles were assembling and forming clusters at the cell surface of HTLV-1-infected T-lymphocytes, and not in uninfected cells. This study showed for the first time that a virus could be associated with a biofilm [94]. Moreover, many T-cell types grow in cluster reflecting the expression of cell surface adhesion molecules. Pais-Correia *et al.* showed also that proteins such as collagen and fibronectin were overexpressed in infected cells, and that Agrin, HSPG and Tetherin were concentrated in the extracellular viral assemblies. Then, they described that galectin-1 and galectin-3 were also upregulated in HTLV-1- infected cells, but galectin-3 was not clustered with the virions. The role of this cellular components is not yet elucidated, neither their processes leading to the synthesis and regulation of the viral biofilm. Alternatively, HTLV-1 could be itself involved in the regulation of these components, since it is well stablished that Tax HTLV-1 can induce expression of many components, such as ICAM-1 and others cell adhesion molecules [91, 94].

Although many works still need to be done towards the understanding of the role of the viral biofilm's components, Dr. Maali *et al.* proposed a model for the viral biofilm formation (**Figure 9**). Thus, it is assumed that when a cell is infected by HTLV-1, there is expression of viral proteins, such as Tax, that could promote the remodeling of extracellular matrix components, with for example an increase of their expression. In parallel, the continuously production of HTLV-1 particles by infected cells leads to their release outside of the cells, where they could stay embedded in the viral biofilm, due to the adhesive capacities of this structure. However, the detailed mechanisms of such viral release are not described. Finally, these viral particles may be released from the biofilm as free particles into the extracellular environment or transmitted to a target cell during the cell-to-cell contact allowing a more effective and rapid transmission [91].

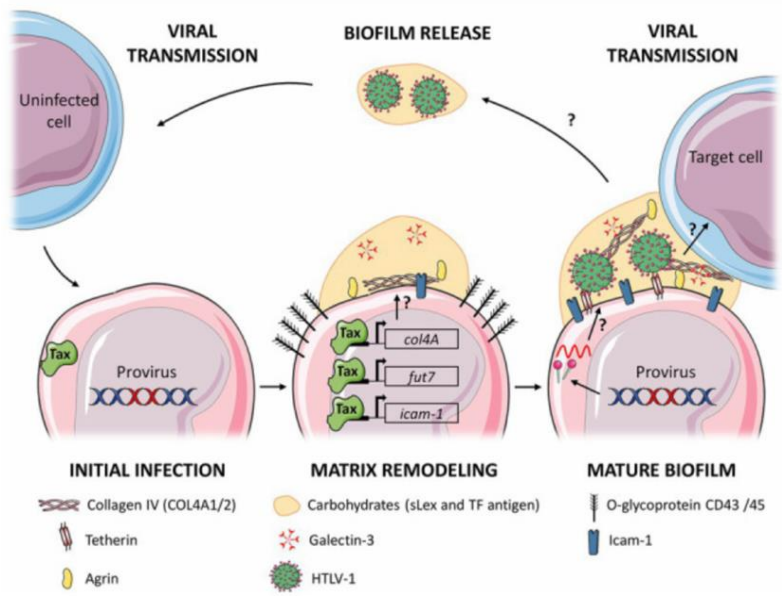


Figure 9 – Proposed models for the stages of HTLV-1 biofilm formation in infected human T-lymphocytes. Image retrieved from Maali Y. *et al.* Frontiers in Microbiology 2020 [91].

This new route of transmission seams then to be important for the infected cells, since this polymeric structure is a big challenge for the innate and adaptive immune system [77, 94, 95]. The infection of a cell by a biofilm is very efficient since this structure is highly concentrated in viral particles and it is directly in contact with the target surface membrane. Viral biofilms can overlap cell-cell contacts and bond the gap between both cell surfaces, rather than filling contact sites. Furthermore, it can also act like a physical protection for the viral *Env* proteins and avoid recognition by host antibodies [77]. Until now, HTLV-1 is the only virus associated to a viral biofilm, and there is not much information about the role of each protein involved either in biofilm formation or during biofilm viral transmission.

3.3. Previous results

Dr. Yousef Maali, at the end of his post-doctoral studies in the Retroviral Oncogenesis team, under the guidance of Dr. Hélène Dutartre began to develop a research (not published) on the characterization of the HTLV-1 biofilm. HTLV-1 infected cell lines (C91-PL, MT-2 and Hut102) and uninfected cell lines (Jurkat) were used in this study. Dr. Maali demonstrated that viral transmission, as well as HTLV-1 infectivity is variable according to the type of cell lines used, correlate this finding to the composition of the biofilm present in each cell line. For example, it has been described that C91-PL cells produce more infectious viral particles than MT-2 cells [36]. In addition, Dr. Maali obtained that viral transmission is also higher in C91-PL cells than in the other lines, and this difference is not related to a difference in viral particle production, since Futsch *et al.* demonstrated, in a previous work, that after an analysis by qRT-PCR, MT-2 cells show higher viral production than C91-PL cells (**Figure *10***) [96]. Furthermore, the density of viral particles in the

biofilm of each cell type was observed, and once again C91-PL shows higher viral density at the cell surface than the other two cell lines **(Figure 11a)**. The cells were then sent to Pr. Philippe Roingeard's team at Tour University to confirm the extracellular localization of the virus by electron microscopy (SEM and TEM). The images showed that in C91-PL cells, viral particles are in greater quantity at the cell's surface and more polarized than in other cell lines **(Figure 11b)**. With all these results it was possible to infer that the different cell lines have different viral production and transmission values. The C91-PL cells produce more infectious particles, which are found in higher density and polarized at the extracellular surface of the cells. Using SEM it is possible to observe the aggregation of viral particles. This aggregation may be related to the viral biofilm. Thus, the present results support the fact that the biofilm composition varies between cell types, which could contribute to different adhesive capacities and consequently variations in viral density at the cell surface.

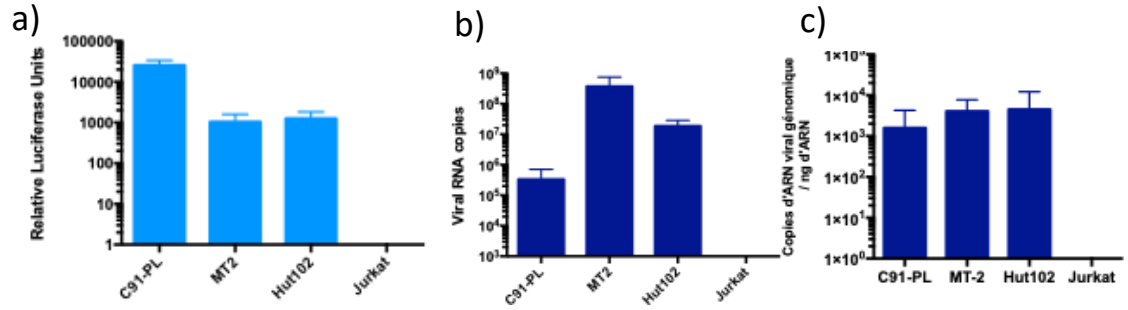


Figure 10 - HTLV-1 transmission by cell contact is most efficient from C91-PL lines. **(a)** HTLV-1 infected cells (C91-PL, MT-2 and Hut102) or uninfected cells (Jurkat) used as controls were cultured with Jurkat reporter cells expressing luciferase under the control of the HTLV-1 LTR promoter (0.2:1 ratio). Luciferase activity was measured after 24 hours of co-culture. Results are represented in RLU on a log scale. **(b)** Viral production by HTLV-1 infected lines quantified in culture supernatant from HTLV-1 infected cells (C91-PL, MT-2 and Hut102) or uninfected cells (Jurkat) used as controls was harvested after 24 h and viral RNA measured by qRT-PCR. **(c)** HTLV-1 infected cells (C91-PL, MT-2 and Hut102) or uninfected cells (Jurkat) used as control) were lysed and viral RNA detected by qRT-PCR.

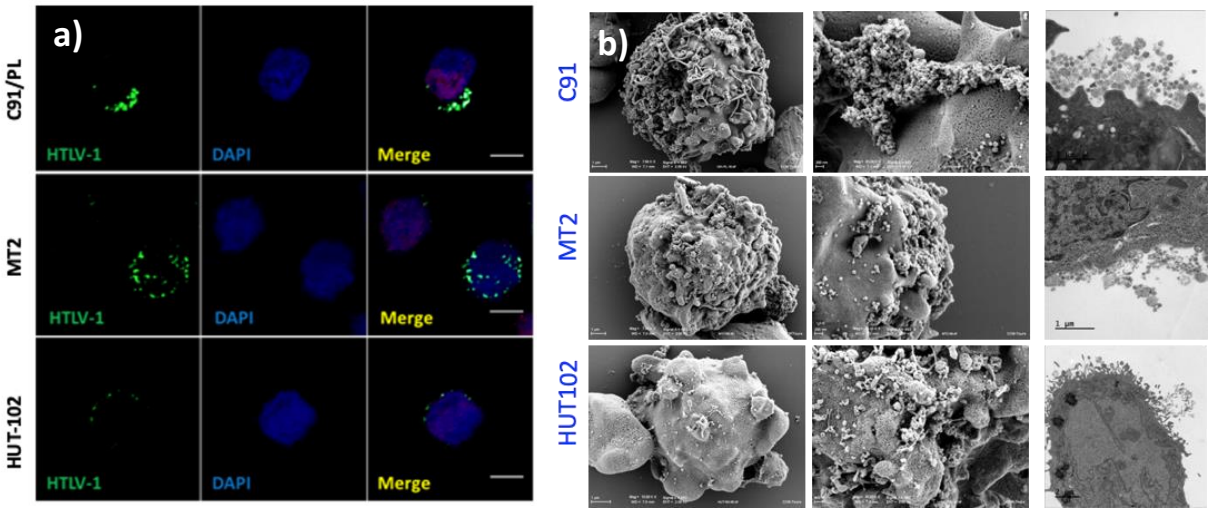


Figure 11 - HTLV-1 virus is localized on the surface of infected cells in a biofilm of varying size depending on the lineage. (a) HTLV-1 infected cells (C91-PL, MT-2 and Hut102) were fixed and then labelled with human serum from an HTLV-1 infected individual followed by labelling with FITC-coupled anti-human antibody. The cells were observed under a confocal microscope. Scale = 5µm. **(b)** HTLV-1 infected cells (C91-PL, MT-2 and Hut102) were fixed

and visualized by scanning electron microscopy (SEM) (first two panels) and transmission electron microscopy (TEM) (last panel). Scale = 1µm.

Then, Dr. Maali went on to observe the composition of the biofilms present in the different cell lines, by analyzing proteins such as Tetherin, ICAM-1, Agrin, and Galectin-3, that appeared to be part of the viral biofilm, as well as Collagen IV and Mucin-1 that were found highly upregulated in infected cells and involved in ECM. Using confocal microscopy (data not showed), it was possible to confirm that ICAM-1, Galectin-3, and Tetherin proteins were co-localized with viral particles, in C91-PL and MT-2, but not in HUT102, which miss Galactin-3. Although, the team observed that Agrin, Mucin-1 and Collagen IV were highly expressed in infected cells, they were not co-localized with the viral particles. These results also showed different abundance of these proteins in the viral biofilm, with C91-PL cells having the highest concentration (**Table *1***). Then, Dr. Maali analyzed the localization of other two proteins at the surface of different infected cell lines. By confocal microscopy it was possible to confirm that Caveolin-1 was upregulated 500-2500-fold as well as the SNAP25, which was upregulated 400-1200-fold in infected cell lines when compared to non-infected T cells. These two proteins are known to be involved in vesicular trafficking and were suspected to be involved in polarized budding of newly synthesized virions. Confocal images (**Figure *12***) showed that both SNAP25 and Caveolin-1 were co-localized with the viral particles in both C91-PL and MT-2 cell lines.

	Galectin-3	ICAM-1	Tetherin	Polarization
C91/PL	+++	+++	+++	+++
HUT-102	-	+	++	-
MT2	+	+	++	-

Table 1 : Schematic table of the results of the analysis of proteins (Galectin-3, ICAM-2 and Tetherin) localized in clusters on the surface of HTLV-1 infected cell lines. HTLV-1 infected cells (C91-PL, HUT-102 and MT-2) were fixed and labelled with specific antibodies for each protein. Visualization of the HTLV-1 viral biofilm was performed by confocal microscopy. C91-PL cells showed high fluorescence intensity for all three proteins and biofilm polarization. The other two cell lines did not have a polarized distribution. HUT-102 and MT 2 cells showed significant levels of Tetherin and low levels of ICAM-1 expression. Galectin-3 expression was also low in the MT 2 cell line but absent on the surface of HUT-102 cells.

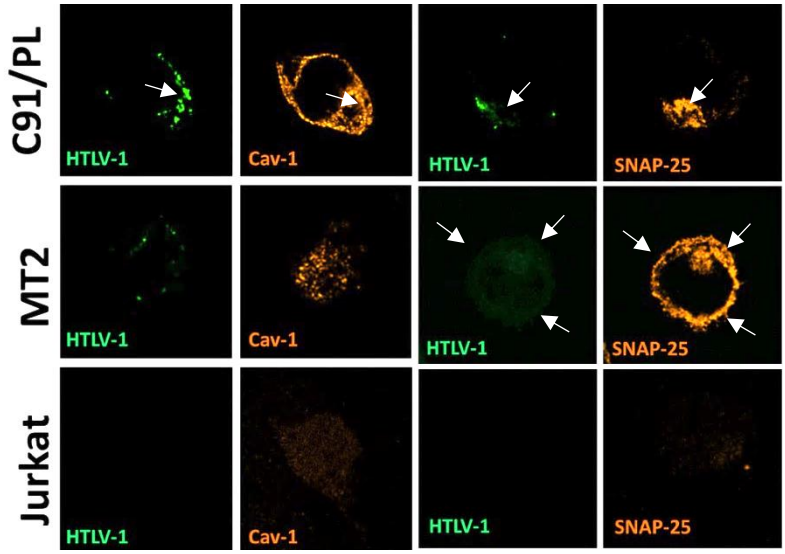


Figure 12 - HTLV-1 virus is localized on the surface of infected cells and there is an upregulation of Caveolin-1 and SNAP25 in these cell lines. HTLV-1 infected cells (C91-PL and MT-2) were fixed and stained with human serum from an HTLV-1 infected individual followed by labelling with specific antibodies for each protein. Uninfected cells (Jurkat) were used as control with the same staining. Arrows represent co-localization of viral particles with Caveolin-1 or SNAP25 protein. The cells are observed under a confocal microscope. Scale = 5µm.

The combination of these results suggests that the density and concentration of virus at the surface of infected cells, as well as the composition and polarization of viral biofilms, are related with the ability of these cells to transmit the virus. The first part of my thesis work was then to pursue the characterization of the composition of the viral biofilm using imaging of different cellular proteins (Caveolin-1 and SNAP25) within the viral biofilm, as well as the study of the role of these proteins in viral transmission to target cells.

3.4. Surface receptors and proteins

In the study conducted by Dr. Yousef Maali, it was possible to identify three proteins that showed co-localization with viral particles and two other proteins that had their expression increased in HTLV-1 infected cells. Therefore, in this project we will focus our attention on the study and characterization of Caveolin-1 and SNAP25 in biofilm-associated viral transmission.

3.4.1. Caveolin-1

Caveolin-1 is multifaceted functional protein composed of 177 amino acid residues that is located in the inner leaflet of the plasma membrane. This protein is essential for caveola formation, an invaginated structure of about 50-80 nm on the membrane, one of the pathways for endocytosis [97]. This 21 to 24kDa protein is involved in a wide range of cellular processes such as exocytosis, cell cycle regulation, cell signaling, and even cell senescence/death [98, 99]. Caveolin-1 is also involved in the regulation of extracellular vesicles exocytosis and cargo selection in many cells [100]. HIV-1 viral binding requires both

cytoskeletal rearrangements and actin associated proteins that interact with Caveolin-1. Although it is not clear which role Caveolin-1 has during viral synapse, it seems that is it important for immunological synapse during for T cell activation [101]. Although Caveolin-1 is expressed in many cell types, lymphocytes express very low levels. Curiously, it is known that HIV-1 infected cells overexpress Caveolin-1 due to the mediation of the Tat protein (an analogous protein of the HTLV-1 Tax), suggesting that Caveolin-1 is important for the viral life cycle. First studies demonstrated that HIV-1 Env proteins bind strongly with Caveolin-1 within infected macrophages. This interaction could suppress membrane fusion, restrict HIV-1 production and reduce infectivity [102, 103]. This upregulation of Caveolin-1 promoting latent infection happens due the changing of cholesterol composition, which decreases fusion and at the same time triggers a danger signal that represses HIV-1 transcription [98, 103]. Therefore, HIV-1 infection upregulates Caveolin-1 during infection, which subsequently suppresses viral replication, by promoting blocking of the virus' fusion steps.

Interestingly, it known that HTLV-1 infected T-cells, as other cell types, have an overexpression of Caveolin-1, that like HIV-1, is promoted by HTLV-1 Tax protein [104, 105].

Yet, it is still unknown if in HTLV-1 infected cells Caveolin-1 promotes the same blockade of HIV-1 infection. During transmission by virologic synapse, HTLV-1 virions can enter into the target cells by endocytosis after being trapped in a caveolar compartment. So, Caveolin-1 seems to be important for viral transmission, at least for HIV-1, making this protein a good candidate to be involved in the HTLV-1 transmission and probably present in the viral biofilm [88].

3.4.2. SNAP25

SNAP25 (synaptosomal-associated protein of 25 kDa) belongs to the SNARE complex which is essential for the vesicular exocytosis depended on Ca^{2+}. SNAP25 binds to lipids via palmitoylation of multiple side chains acting as an important linker for the SNARE complex, both accelerating the complex assembly and interacting with the local lipids, in order to preserve the membrane configuration and to facilitate vesicle fusion [106]. Additionally, it is well stablished that SNAP25 plays a crucial role during neuronal synapse formation [107]. Taking into account the importance of SNAP25 during exocytosis and endocytosis it is conceivable that SNAP25 repression will affect virus entry, as it was already described for the adenovirus [108]. HIV-1 can contribute to many neurological comorbidities. Although neurons are not easily infected by HIV-1, these cells are affected by the cellular factor Tat [107, 109]. Eletto *et al.*, demonstrated that SNAP25 expression is deregulated by HIV-1 Tat protein. On the other hand, within T-cells, the role of this protein is still not well elucidated.

In addition, it is known that some proteins from the SNARE complex are involved in both immunological and virological synapse formation and consequently in HIV-1 cell-to-

cell spread [110, 111]. It was observed that, for example, STX4 (syntaxin-4) protein from the SNARE complex was involved in cell-to-cell spread. The silencing of this protein reduced the viral transmission by contact between cells, which was observed both by qPCR and luciferase assay. Moreover, the quantification of viral particles in the supernatant reveled that the reduction of viral transmission was not due to the cell free particles. This group also observed that there was lower formation of virologic synapses once STX4 expression is reduced. Hence, it is possible that STX4 protein regulates cell-to-cell spread of HIV-1 thought the virological synapse [110]. Furthermore, Jain *et al.*, showed also that some proteins from the SNARE complex, such as SNAP25, had significant increase of their expression in HTLV-1-transformed C8166 T-cells compared with non-infected Jurkat cells [112], a complementary result to those obtained by Yousef Maali (**Figure *10***). All these results suggest that proteins within the SNARE complex could be involved in both HIV-1 and HTLV-1 replicative cycle.

3.5. Summary

HTLV-1, contrary to HIV-1, is transmitted mostly through cell-cell contact, and its viral particles are weakly infectious *in vitro*. Currently, it is known that this virus can be transmitted by three different forms of cell-cell contact. The first mode of transmission involves the formation of cellular conduits: long elongations of actin that allow cell-cell communication and transmission of virions. HTLV-1 transmission can also occur by virological synapse, similar to the immunological synapse. This transmission route requires close contact between two cells with formation of a synaptic cleft. In addition, HTLV-1, to date, is the only virus that has a third described transmission route: the viral biofilm. Biofilm is a hydro-polymeric extracellular matrix composed of adhesive molecules and many other protein types. During years, this type of structure was a feature of bacteria and fungi. As for bacteria, the biofilm allows the protection of the viral particles from the immune system, as well as the retention of a greater number of viruses in the same space, which consequently allows a more efficient viral transmission through cell-cell contact.

Unpublished studies have confirmed that the density and concentration of viruses on the surface of infected cells, as well as the composition of the biofilm, are related to the capacity of viral transmission. Furthermore, it has been observed that there is a polarization of the viral biofilm, i.e. it has been found that both viral particles and some cellular molecules/receptors are co-localized and polarized at the same time. This suggests that some molecules may play an important role in biofilm formation and/or viral transmission. So far, ICAM-1, Tetherin, Galectin-3, Caveolin-1 and SNAP25 are candidates to exist in the viral biofilm.

Caveolin-1, as well as SNAP25 have their expression increased in HTLV-1 infected cells. It has already been described that both Caveolin-1 and SNAP25 are important in the processes of endo- and exocytosis of viral particles. Caveolin-1 protein also plays a role in virological synapse formation of HIV-1. On the other hand, SNAP25 is a SNARE complex protein important for HIV-1 virological synapse formation, as well as for both neural and immunological synapses. Due to the similarity between HIV-1 and HTLV-1, many processes occur similarly and using the same types of molecular players, so it is possible that Caveolin-1 and SNAP25 are also involved in viral transmission of HTLV-1, for which the expression of both these proteins is increased in infected cells.

Chapter 4

Material and Methods

4.1. Cells

HEK 293T cells and HeLa cells were cultivated in DMEM (Dulbecco's Modified Eagle's Medium, Gibco, Invitrogen, 61965-026) supplemented with 10% fetal calf serum (FCS; Gibco Life Technologies), 100μg/mL penicillin-streptomycin (P/S; Gibco Life Technologies) and 1% Sodium Pyruvate (Invitrogen, 11360-039). The two non -infected cell lines Jurkat cells (from ATCC) and Jurkat LTR-Luc, as well as two infected cell lines C91-PL (HTLV-1 infected T-cell line, CVCL_0197) and MT-2 (Human cord leukocyte cell line established by co-cultivation with human ATL cells) were maintained in RPMI 1640 medium (RPMI1640 GlutaMAX, Invitrogen, 61870-010) supplemented with 10% FCS and P/S (100μg/mL). All cells were grown at 37ºC and 5% CO_2. Jurkat LTR-Luc are transfected cells with a plasmid encoding luciferase protein under the control of the HTLV-1 LTR promoter. These cells were maintained under hygromycin (450μg/mL, Sigma) selection.

4.2. Antibodies

Table 2: Sum-up of the antibodies with respective dilutions used.

Antibody	Specie	Dilution		Supplier, Reference
		Western Blot	IF & FACS	
Anti-beta-actin	Mouse	1:6000	-	Sigma, A2228
Anti-p19	Mouse	1:400	-	Zeptometrix, 801003
Anti-mouse	Sheep	1:40000	-	NA9310V
Anti-SNAP25 (coupled with Alexa Fluor 647)	Mouse	1:500	1:100	Santa Cruz Biotecnology, sc-20038
Anti-Caveolin-1 (coupled with Alexa Fluor 594)	Mouse	1:500	1:100	Santa Cruz Biotecnology, sc-53564
Human Serum HTLV-1+	Human	-	1:400	Gift from Dr. Gessain (Institut Pasteur)
Anti-human (Alexa Fluor 488)	Goat	-	1:1000	Vector, DI-3488

4.3. Plasmid's amplification and purification

Transformed bacteria with the respective plasmid for each small hairpin RNA (shRNA) (**table 3**) were plate in LB-agar medium with 1:1000 of ampicillin during 48h. Bacteria colonies were isolated and placed in 200 ml of LB medium supplemented with 200μL of ampicillin. Bacteria amplification occurred over night at 37ºC. Plasmid DNA purification was performed by Maxiprep according to the supplier's instructions (Macherey-Nagel) and the quantification was done by Nanodrop.

4.3.1. Sequencing

After purification, all plasmids were sent to sequencing in order to verify the presence of the shRNA genome. The sequencing was performed by GATC society, and the sequence analysis were performed resourcing to Databases like Crustal Omega, EMBL and BLAST, NCBI.

Table 3: Plasmids used for the production of the lentivectors.

Gene	Plasmid name	Region	Sequence
Caveolin-1	shRNA Caveolin-1_ 00	CDS	CCGGCCACCTTCACTGTGACGAAATCTCGAGATTTCGTCACAGTGA AGGTGGTTTTT
Caveolin-1	shRNA Caveolin-1_ 02	CDS	CCGGGACGTGGTCAAGATTGACTTTCTCGAGAAAGTCAATCTTGAC CACGTCTTTTT
Caveolin-1	shRNA Caveolin-1_ 11	3UTR	CCGGGCTTTGTGATTCAATCTGTAACTCGAGTTACAGATTGAATCA CAAAGCTTTTTG
Caveolin-1	shRNA Caveolin-1_ 18	CDS	CCGGGACGTGGTCAAGATTGACTTTCTCGAGAAAGTCAATCTTGAC CACGTCTTTTT
Caveolin-1	shRNA Caveolin-1_ 99	3UTR	CCGGGCTTTGTGATTCAATCTGTAACTCGAGTTACAGATTGAATCA CAAAGCTTTTT
SNAP25	shRNA SNAP25_22	CDS	CCGGCGATACACAGAATCGCCAGATCTCGAGATCTGGCGATTCTGT GTATCGTTTTTTG
SNAP25	shRNA SNAP25_25	CDS	CCGGCGATACACAGAATCGCCAGATCTCGAGATCTGGCGATTCTGT GTATCGTTTTTG
SNAP25	shRNA SNAP25_30	3UTR	CCGGCCTTGATGTCTTGAGTTTCATCTCGAGATGAAACTCAAGACA TCAAGGTTTTTTG
SNAP25	shRNA SNAP25_36	CDS	CCGGGAACCATATCAACCAAGACATCTCGAGATGTCTTGGTTGATA TGGTTCTTTTTTG
SNAP25	shRNA SNAP25_72	3UTR	CCGGCCCAACAATACTTTGTGTCTTCTCGAGAAGACACAAAGTATT GTTGGGTTTTTTG

4.4. Lentivectors production

Lentivectors were produced for each plasmid. HEK293T cells ($7x10^6$ cells) were transfected with 40uL of 8.2 plasmid, 20uL of VSVg plasmid and 40uL of the shRNA plasmid correspondent (Table 3) or GFP plasmid (as control). Plasmids were precipitated using $CaCl_2$ and HBS and then added to the cells. Supernatant was harvest after 48h and filtered thought a 0.45 um-diameter pore filter (Millipore) to eliminate the remain cells. Lentivectors were purified by ultracentrifugation thought a 20% (wt/vol) sucrose cushion at 28,000 rpm for 1h30min at 4ºC. Viral pellet was resuspended in 100 μL of RPMI 1640 medium without S/P and FCS.

4.4.1. Reverse Transcriptase assay

For the quantification of the lentivectors, 5μL for each condition were plate in a 24-well plate and analyzed by reverse transcriptase (RT) assay.

4.4.2. Lentivectors titration

HeLa cells ($1x10^5$ cells) were plate with DMEM medium in a 12 well-plate. Once cells were adhered, GFP lentivectors were added to the respective well at a serial dilution: 10μL, 1μL, 0,1μL and 0 μL (negative control). After 72h, cells were collected, fixed with 4%

paraformaldehyde (4% PFA), and washed with PBS 1X. Cells were analyzed by flow cytometry.

4.5. Cell transduction and protein silencing

C91-PL and MT-2 ($1x10^6$ to $1,5x10^6$cells) were plate in a 24-well plate with 0,5 mL of RPMI medium. Lentivectors were added in the indicated multiplicity of infection of 1 (MOI=1). Medium was changed after 4 hours. Cells were maintained in culture at 37ºC until analysis and the medium was changed every 2 or 3 days.

Three transductions were performed at different time points. Different combinations of the lentivectors were performed for both Caveolin-1 and SNAP25 silencing. The first transduction was performed in C91-PL cells, which were transduced with the combination of lentivectors: shCaveolin-1_1, shSNAP25_1, shSNAP25_2, shSNAP25_All, GFP. In the second transduction, we used C91-PL, and MT-2 cell lines transduced with shCaveolin-1_All, shSNAP25_All and GFP. Moreover, for all the transductions wild type (WT) cells were maintained in the same conditions. Cells were collected and analyzed at different days after transduction.

Table 4: Lentivectors combination

Name of the combination	Lentivectors	Plasmid of reference
shCaveolin-1_1	LV_shRNA Caveolin-1_ 00	shRNA Caveolin-1_ 00
	LV_shRNA Caveolin-1_18	shRNA Caveolin-1_18
	LV_shRNA Caveolin-1_99	shRNA Caveolin-1_99
shCaveolin-1_All	LV_shRNA Caveolin-1_00	shRNA Caveolin-1_00
	LV_shRNA Caveolin-1_02	shRNA Caveolin-1_02
	LV_shRNA Caveolin-1_18	shRNA Caveolin-1_18
	LV_shRNA Caveolin-1_99	shRNA Caveolin-1_99
shSNAP25_1	LV_shRNA SNAP25_22	shRNA SNAP25_22
	LV_shRNA SNAP25_25	shRNA SNAP25_25
	LV_shRNA SNAP25_36	shRNA SNAP25_36
shSNAP25_2	LV_shRNA SNAP25_25	shRNA SNAP25_25
	LV_shRNA SNAP25_36	shRNA SNAP25_36
	LV_shRNA SNAP25_72	shRNA SNAP25_72
shSNAP25_All	LV_shRNA SNAP25_22	shRNA SNAP25_22
	LV_shRNA SNAP25_25	shRNA SNAP25_25
	LV_shRNA SNAP25_36	shRNA SNAP25_36
	LV_shRNA SNAP25_72	shRNA SNAP25_72
GFP	LV_GFP	GFP

4.5.1. FACS analysis

For Caveolin-1 and SNAP25 staining, cells were collected, fixed and permeabilized in buffer, provide by eBioscience™ (FOXP3/Transcription Factor Staining Buffer Set, Invitrogen, 00-5523-00) and stained with an anti-Caveolin-1 coupled with Alexa Fluor 594 (Santa Cruz Biotecnology, sc-53564) and anti-SNA25 coupled with Alexa Fluor 647 (Santa Cruz Biotecnology, sc-20038) antibody. In some experiments, cells were previous stained with human serum HTLV-1+ followed by an antibody anti-human. While samples stained for HTLV-1 particles and Caveolin-1 were acquired on a BD FACSCantoII Flow Cytometer, samples stained for SNAP25 were acquired on MACSQuant VYB Flow Cytometer. FACS results were interpretated using FlowJo_V10 Sofware.

4.5.2. Immunofluorescence and Confocal Microscopy

Transduced cells were plated in Lab-Tek chamber (Nunc) pretreated with Poly-L-lysine (Sigma). Cells were fixed with 4% paraformaldehyde (4% PFA), washed with PBS 1X, neutralized in NH_4Cl 50 mM solution, and were stained with human serum HTLV-1+ (gift from Dr. Gessain, Institut Pasteur), followed by goat anti human antibody. After washes in PBS, cells displayed in Dapi-FlouromountG (Southern Biotech). Samples were analyzed by AxioImager (Zeiss) microscope, and results were treated using ImageJ.

4.5.3. Protein quantification

Lysate of proteins were added to 40uL of Protein Assay Dye Reagent 5X (Bio-Rad, 5000006) with 160 uL of water. After homogenization, solution was transferred to a 96-well plate. Protein quantification was measured by the absorbance at 590nm acquired in a Mithras spectroscopy (Berthold, Mithras LB 940 Multimode Microplate Reader). Standard curves were obtained by graphs of light absorbance versus a standard of concentrations of albumin (BSA) 0,1ug/uL to 2ug/uL.

4.5.4. Western Blot analysis

Transduced cells were stored at -80ºC, lysed by RIPA (50mM of Tris, 150 mM of NaCl, 1% of NP-40, 0,25% of NaDoc and 79,5% H_2O) solution and protein concentration was measured by Bradford method, in order to obtain 15-40 ug of proteins. Samples were denaturated at 95ºC in the presence of reductor agent 1X (Bio-Rad, 1610792) and a buffer 1X (Bio-Rad, 1610791).

Proteins were loaded on a polyacrylamide gel (10%) within MEMS tampon and separated by migration (50V-200V, 1h30). Then, proteins were transferred on a PVDF membrane pre-activated in ethanol tampon. After saturation, membrane was emerged in a bath with Caveolin-1 or SNAP25 antibody and followed by a bath with the secondary antibody (anti-mouse). Then, membrane was subject to p19 and actin antibodies. Membrane is incubated in

a detection solution kit Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, RPN2232) for 2 minutes. Finally, membrane was reveled in a Optimax system.

4.6. Cell-free viral particles

The supernatant of each transduced cell was collected at day 7 and 14 after the second transduction and frozen at -80ºC until analysis.

4.6.1. Elisa

Quantification of p19 protein will be performed according to the manufacturer's instructions (Zeptometrix, 0801116). (See Supplementary data 1)

4.7. Co-culture of infected and reported cells

Jurkat-LTR-Luc cells were cocultured (ratio of 5:1) with the HTLV-1 infected T-cell lines (MT-2 and C91-PL) after Caveolin-1 and SNAP25 silencing. Coculture of Jurkat-LTR-Luc cells with C91-PL wild type (WT) cells or Jurkat cells were used as positive and negative controls, respectively. After 24h, cells were collected, and reporter activities were assessed using the luciferase reporter assay system (Promega).

4.7.1. Luciferase assay

Cells in co-culture were centrifuged and pellets were lysed with 100uL of a lysis buffer (Passive lysis buffer, Promega, E1941). Luciferase activity was measured by chemiluminescence from 40uL of the lysate with 80uL of substrate (LAR, Promega, E1501) within a GloMax Microplate Reader (Promega, E6501). Protein quantification was realized by Bradford method.

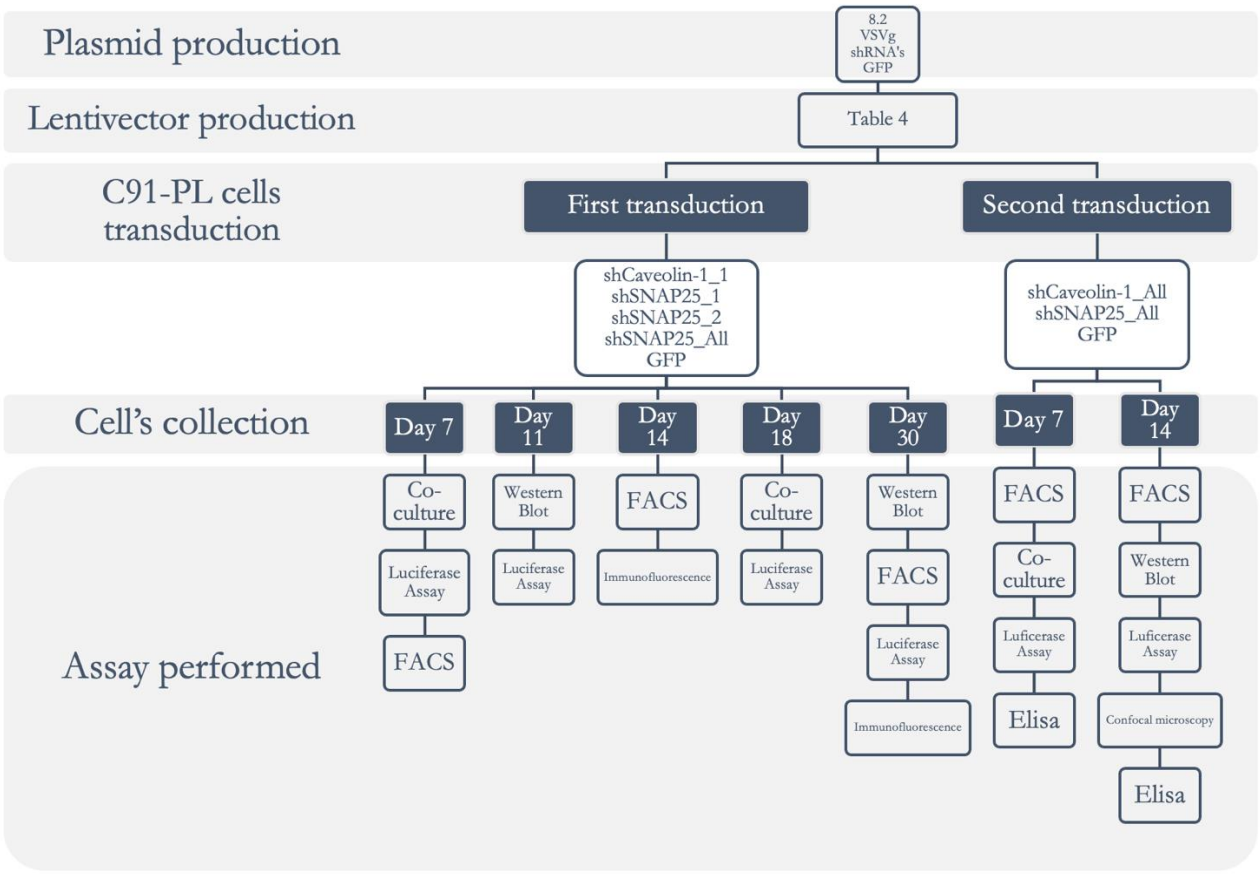
4.8. Statistical analysis

Data was analyzed in Graphpad Prisma (GraphPad Software Inc.) using paired Student's t test, because we compared two groups, the shCaveolin-1 or the shSNAP25 samples with wild type. Differences between groups were considered statistically significant when $p<0.05$ (* $p<0.05$, ** $p<0.01$, *** $p<0.01$). Statistically not significant values are represented as n.s.

4.9. Ethics statement

The "Comité de protetion des personnes Ile de France" has approved the use of human samples used in this study. CPP-IDF-2012-10-04SC to Dr. Gessain (Institut Pasteur, France).

4.10. Summary


Plasmid production
8.2
VSVg
shRNA's
GFP
Lentivector production
Table 4
C91-PL cells transduction
First transduction
Second transduction
shCaveolin-1_1
shSNAP25_1
shSNAP25_2
shSNAP25_All
GFP
shCaveolin-1_All
shSNAP25_All
GFP
Cell's collection
Day 7
Day 11
Day 14
Day 18
Day 30
Day 7
Day 14
Assay performed
Co-culture
Luciferase Assay
FACS
Western Blot
Luciferase Assay
FACS
Immunofluorescence
Co-culture
Luciferase Assay
Western Blot
FACS
Luciferase Assay
Immunofluorescence
FACS
Co-culture
Luficerase Assay
Elisa
FACS
Western Blot
Luficerase Assay
Confocal microscopy
Elisa

Chapter 5

Results

5.1.1. Evaluating Caveolin-1 silencing efficiency in C91-PL cells

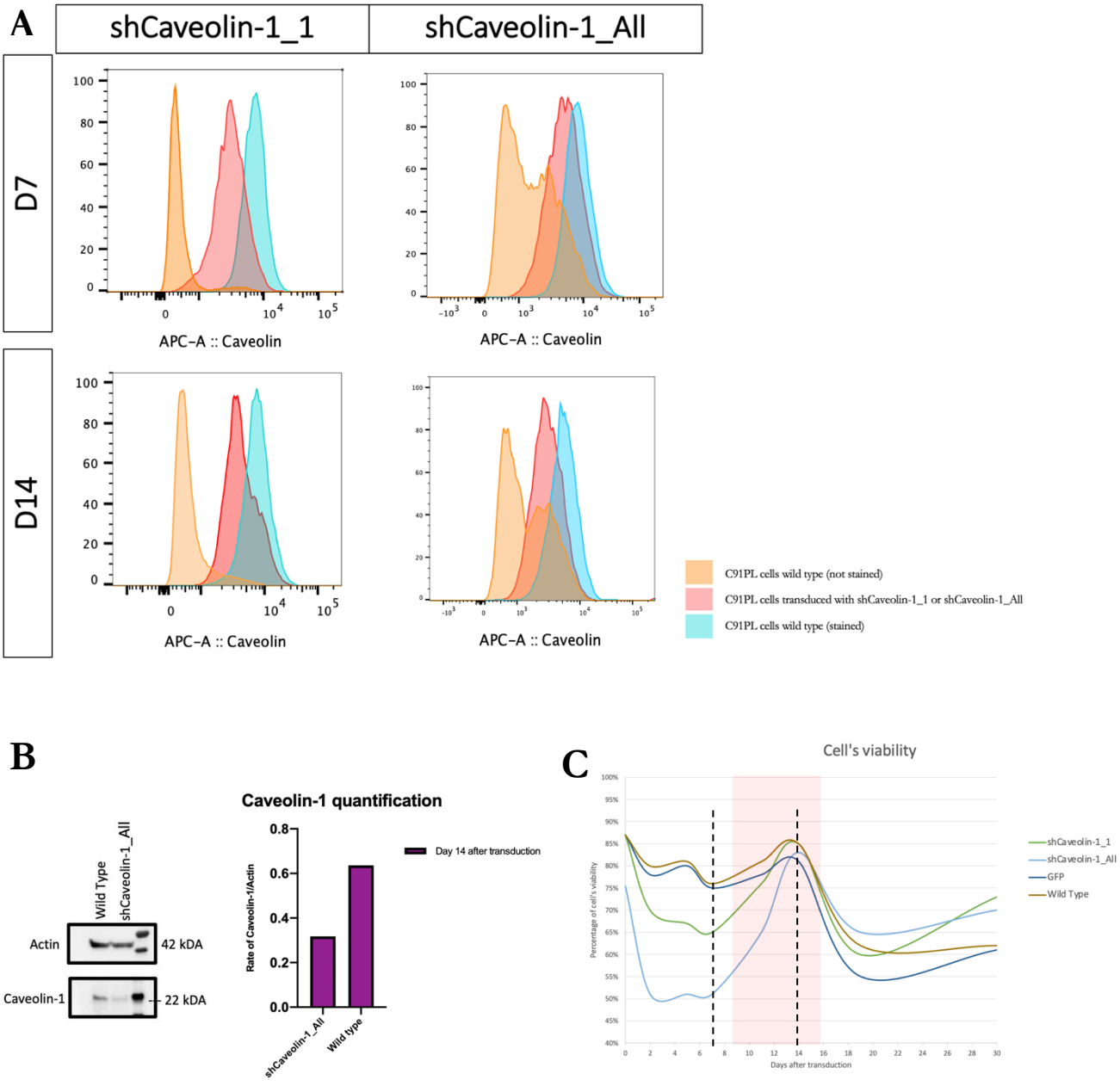


Figure 13 - Evaluating Caveolin-1 silencing efficiency in C91-PL cells. (A) Flow cytometry analysis of C91-PL transduced cells with shCaveolin-1_1 or shCaveolin-1_All, were stained with an anti-Caveolin-1 antibody (red). C91-PL wild type (WT) cells not stained were used as negative control (orange) and C91-PL WT cells stained with the anti-Caveolin-1 antibody were used as the positive control (blue). Cells were analyzed day 7 (D7) and day 14 (D14) after transduction. Results are normalized. **(B)** Western blot analysis of C91-PL cells transduced with shCaveolin-1_1, at D7 (left). C91-PL WT cells were used as positive control. Band's intensity was analyzed by ImageJ software and the rate of Caveoli-1 *per* Actin was represented in a bar graph (right). **(C)** Representative graph of cell's viability over time of C91-PL transduced cells with shCaveolin-1_1 (green line) or shCaveolin-1_All (light blue line). C91-PL cells were also transduced with LV_GFP (control of transduction, dark blue line). Positive control was C91-PL WT cells (brown line). Range of days were cells have high viability (red area) are represented and range where silencing, and cell's viability is optimal is restricted by the vertical lines (dashed lines).

HIV-derived lentivirus vectors encoding small hairpin RNA were used to knockdown the Caveolin-1 protein. This silencing system allows the transcription by cells of a mRNA complementary to the mRNA that codes for the protein Caveolin-1. Once established the complementarity interaction between these two nucleic acids, there is the recruitment of a multiprotein complex named RISC (RNA-induced silencing complex) which promotes the elimination of the target mRNA, and consequently leads to the reduction of the protein expression. In a previous work, I determined that the best way to silence a protein is to use a lentivector for each shRNA sequence (**Table *4***), rather than producing a bulk of lentivectors from transfection with all the shRNA sequences. Each lentivector was then produced using transfection of HEK293T cells with the plasmid coding for a single shRNA sequence, and two other plasmids encoding for proteins involved in the lentivector envelop and packaging. Not all plasmids with the shRNA sequences were used since after sequencing they did not present the sequence of interest. Subsequently, target cells were transduced with 3 or 4 lentivectors, each coding for one shRNA (MOI=1 for each lentivector) resulting in two combinations used for Caveolin-1 protein silencing: the shCaveolin-1_1 condition combined three lentivectors, and the shCaveolin-1_All combined the same three lentivectors and one more. C91-PL cells were transduced (D0) first with shCaveolin-1_1 and in a second transduction with shCaveolin-1_All. In addition, C91-PL cells were also transduced with lentivector allowing expression of GFP (LV-GFP), a condition that served as a transduction control. Viability and cell count was assessed every 2/3 days and Caveolin-1 protein silencing was assessed at day 7 (D7) and day 14 (D14) after transduction, by Flow cytometry and Western Blot. Before D7, cell numbers in culture were decreasing significantly as well as the viability of cells transduced with shCaveolin-1_1 in the first days (**Figure 13C**), preventing the collection of a sufficient amount to perform all the necessary tests. This different behavior does not seem to be associated to the transduction by itself, since cells transduced with LV-GFP did not show alteration in the growing. Thus, it seems that the reduction of Caveolin-1, promotes high damage, and great mortality of the cells in culture.

The efficiency of the silencing was finally observed D7 for the cells transduced with shCaveolin-1_1, and the silencing was maintained at least until D14 (**Figure 13A**). As for the shCaveolin-1_All combination, silencing was greatly reduced (**Figure 13A**). To confirm that Caveolin-1 was efficiently silenced, cells transduced using the shCaveolin-1_All combination were analyzed by Western Blot at D14 **(Figure 13B**). Compared to parent C91-PL cells, transduced cells showed a decreased expression of Caveolin-1 of around 50% as determined by quantification of Western Blot signals. Analysis of the cell's viability graph **(Figure 13C, red area)** showed that cells transduced with shCaveolin-1_All were more damaged with the transduction than the others, despite it really seems that transduction by itself affects the viability. Therefore, this suggests that Caveolin-1 is a vital protein whose complete silencing would lead to cell death.

Next, we observed that the cell viability decreased significantly until 7 days post transduction after which it increased again back to normal rate. To evaluate whether cell viability was correlate to reverted Caveolin-1 expression we collected cells day 18 and 30 (D30) after transduction and we analyzed them by flow cytometry and then by Western Blot.

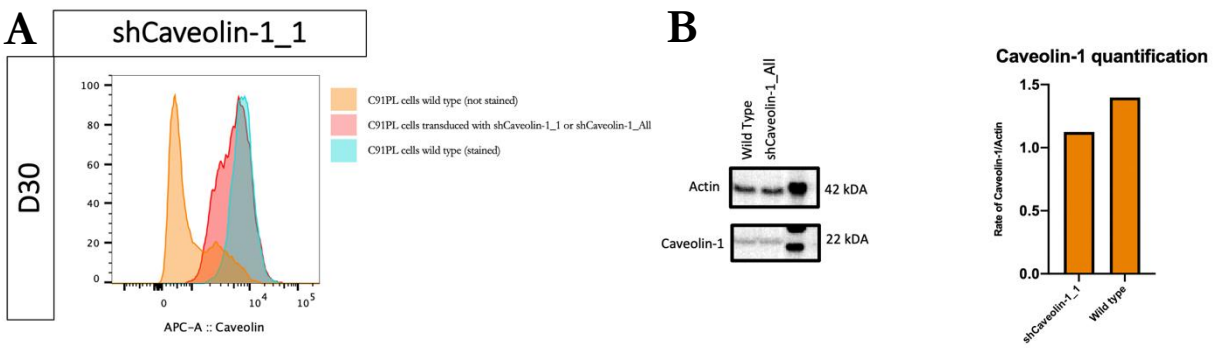
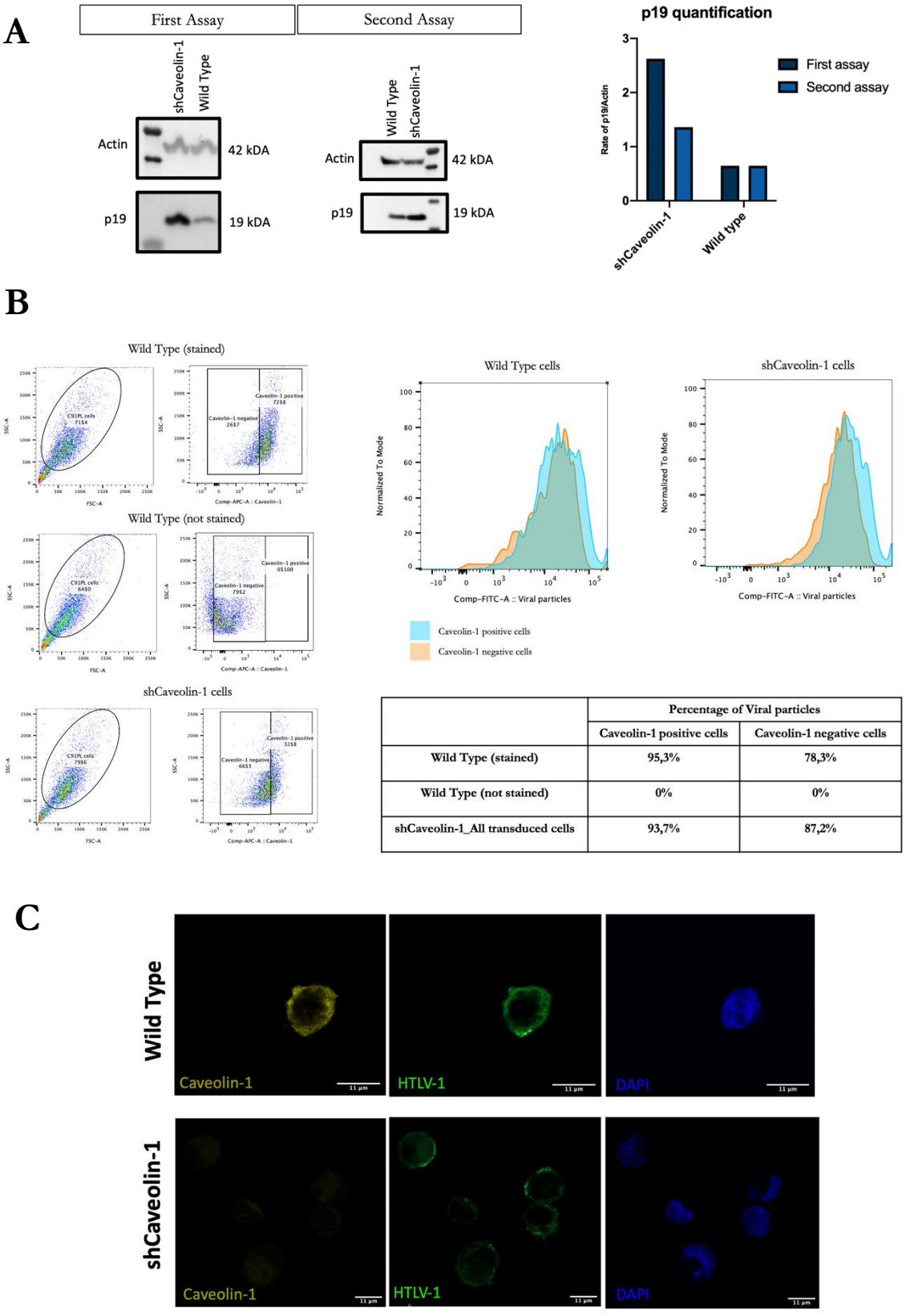


Figure 14 - Caveolin-1 silencing after 30 days of transduction. (A) Flow cytometry analysis of C91-PL transduced cells with shCaveolin-1_1 and stained with an anti-Caveolin-1 antibody (red). C91-PL wild type (WT) cells not stained were used as negative control (orange) and C91-PL WT cells stained with the anti-Caveolin-1 antibody were used as the positive control (blue). Cells were analyzed day 30 (D30) after transduction. Results are normalized. **(B)** Western blot analysis (left) of C91-PL cells transduced with shCaveolin-1_1, at D30. C91-PL WT cells were used as positive control. Band's intensity was analyzed by ImageJ software and the rate of Caveoli-1 *per* Actin was represented in a bar graph (right).

Caveolin-1 expression was reestablished D30 **(Figure 14A and B**). So, it seems that Caveolin-1 silencing induced cell's death but because silencing was not complete, with cells not silenced, we hypothesized that non transduced cells proliferate better and took the place of the others over time. Another simple hypothesis is that silenced cells developed a way to express Caveolin-1 back.

Altogether these results allow to define that using the combination shCaveolin-1_1 is the most efficient and less aggressive for the cells. In addition, transduced cells have to be analyzed between 7-14 days after transduction (**Figure 13C, dashed lines)**.

5.1.2. Viral expression in Caveolin-1 silenced cells



	Percentage of Viral particles	
	Caveolin-1 positive cells	Caveolin-1 negative cells
Wild Type (stained)	95,3%	78,3%
Wild Type (not stained)	0%	0%
shCaveolin-1_All transduced cells	93,7%	87,2%

Figure 15 - Evaluating viral expression in Caveolin-1 silenced cells. (A) Western blot analysis (left) of C91-PL cells transduced with shCaveolin-1 at different timepoints. Membranes were stained with p19 antibody followed by actin

antibody. C91-PL WT cells were used as positive control. Band's intensity was analyzed by ImageJ software and the rate of p19 *per* Actin was represented in a bar graph (right). **(B)** Flow cytometry analysis of C91-PL transduced cells with shCaveolin-1. Cells were first stained with human serum HTLV, permeabilized and stained with anti-Caveolin-1 antibody. C91-PL wild type (WT) cells not stained were used as negative control and C91-PL WT cells stained with the anti-Caveolin-1 antibody were used as the positive control. Plots with the chosen gating's are presented (left) for all the conditions regarding the expression of Caveolin-1. Cells with positive expression of Caveolin-1(blue) and with negative expression of Caveolin-1 (orange) are represented in the histograms (top right) regarding the quantity of viral particles present in each condition. Results are normalized and simplified in a table (bottom right). **(C)** Confocal microscopy was performed in shCaveolin-1 cells (bottom line) and in wild type cells (top line). All cells were stained with anti-Caveolin-1 antibody (yellow), human serum HTLV-1 (green) and DAPI (blue). Scale=11 μm.

We next evaluated the production of viral particles in Caveolin-1 silenced C91-PL cells. First, we determined the level of p19 Gag by western blot 14 days after transduction (**Figure 15A**). Actin staining was used for normalization. Compared to parent cells, the level of p19 in Caveolin-1 silenced cells was higher in the two independent transduction experiments **(Figure 15A comparing left and right panel, first and second assays)**, however, in the second experiment the difference with the parent cells was lower. Quantification of band intensity was normalized to that of actin in all samples, showing an increase of 2 to 4 folds the expression of p19 in Caveolin-1 silenced cells **(Figure 15A, right).** The difference of p19 expression was correlated with the efficiency of silencing: for the first assay, silencing was approximately 70% (observed by flow cytometry) while for the second it was 49,9% (observed by western blot and flow cytometry) (**Figure 13B)**. Therefore, this suggested that the reduction of Caveolin-1 protein is associated with an increase in the amount of p19 within shCaveolin-1 cells, and that when Caveolin-1 expression is re-established, the amount of p19 approaches the amount observed in WT cells (not shown).

The Western blot allows an analysis of the amount of p19 both intracellular and extracellular, if associated to the cellular biofilm. So, next, we wanted to verify the viral expression only at the cell's surface. To that purpose, cells were first labeled with a human serum form an HAM/TSP patient that recognized several HTLV-1 proteins, including Env, then permeabilized and labeled with an anti-Caveolin-1 antibody. Flow cytometry analysis showed that the percentage of surface viruses is similar on cells with low or normal expression of Caveolin-1(**Figure *15* B).** Next, cells were observed by confocal microscopy to confirm the surface level of virus upon Caveolin silencing. Again, Caveolin-1 silencing did not significantly change the intensity of HTLV-1 present in the viral biofilm, while Caveolin signal was decreased compare to that in WT cells **(Figure 15 C).** In addition, it seems that viral distribution is not altered in Caveolin-1 silenced cells as viruses were still polarized **(Figure 15 C).**

5.2. Viral transmission in Caveolin-1 silenced cells

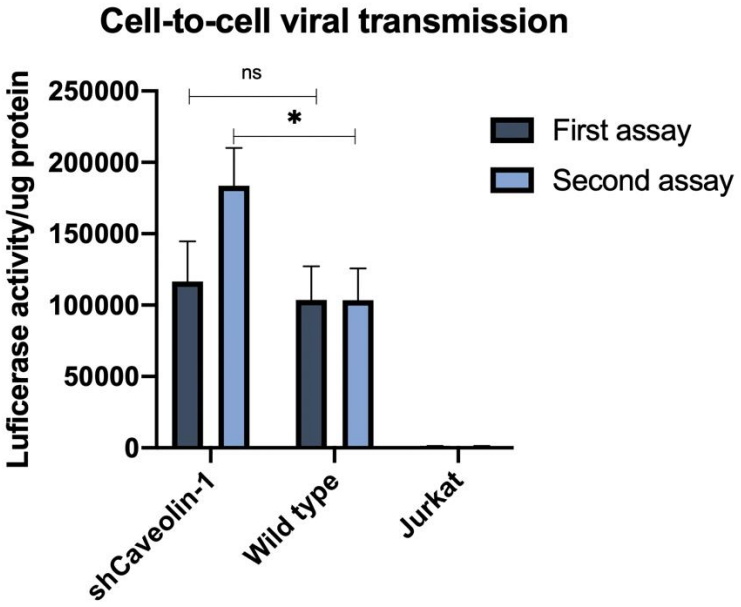


Figure 16 - Evaluation of cell-to-cell viral transmission in Caveolin-1 silenced cells. C91-PL wild type cell, shCaveolin-1 cells and Jurkat cells were co-cultured with Jurkat LTR-luc cells. Viral transmission is proportional to the quantity of Luciferase protein produced by Jurkat LTR-luc cells. Luciferase is quantified by fluorescence and results were presented in a bar graph. First assay: day 7 after second transduction. Second assay: day 14 after second transduction.

Finally, we measured viral transmission in shCaveolin-1, WT C91-PL and Jurkat cells, after co-culturing them with Jurkat LTR-luc reporter cells. If viral transmission occurs, Jurkat LTR-luc cells produce Luciferase protein which can be quantified by fluorescence. In Caveolin-1 silenced cells the luciferase activity was significantly higher compared to WT cells, only in the second independent experiments (**Figure 16**). This difference correlates to the level of silencing at the time of the analysis. Indeed, Caveolin-1 expression was lower for the second repetition.

5.3. Evaluating SNAP25 silencing efficiency in C91-PL cells

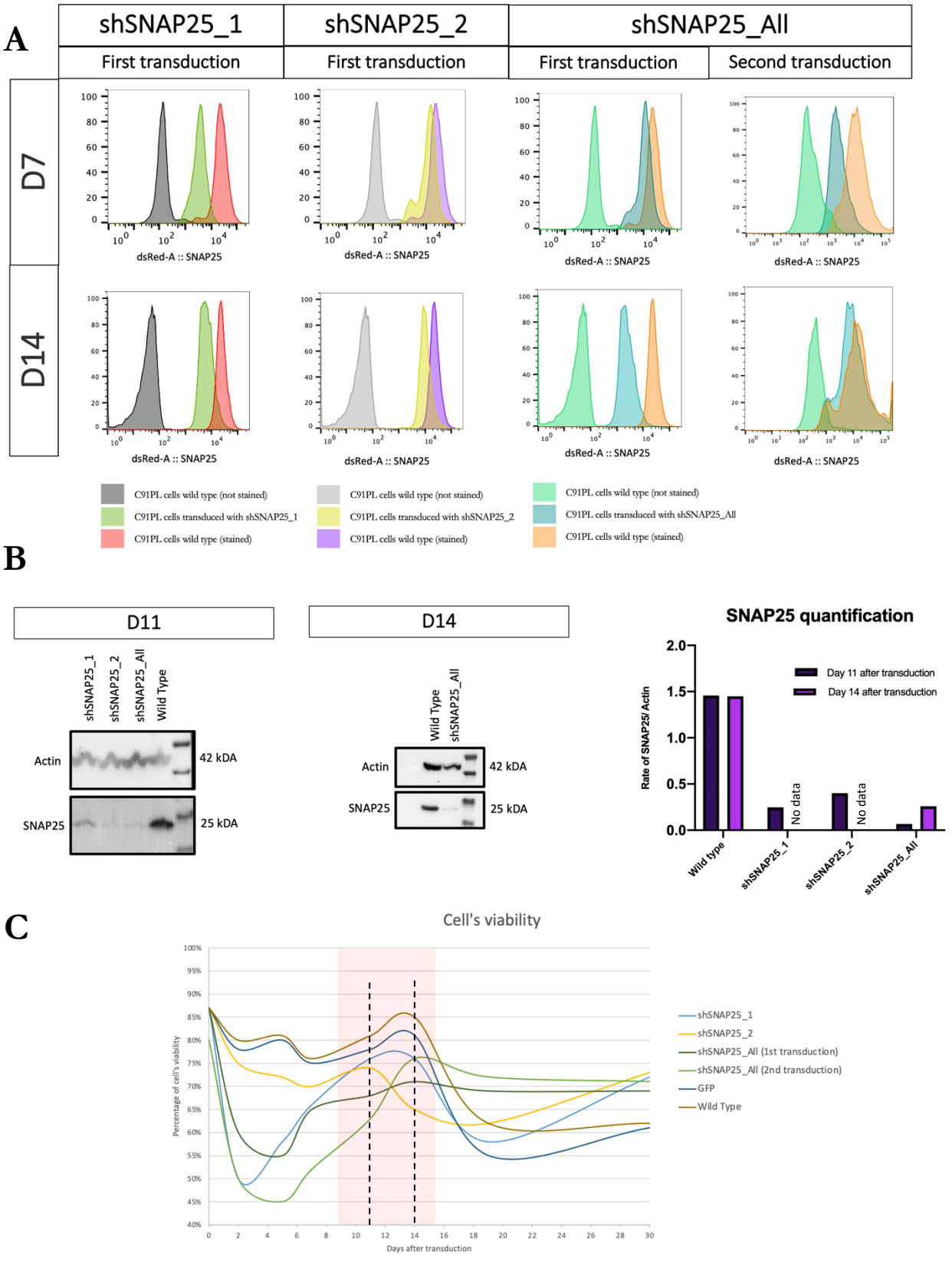


Figure 17 - Evaluating SNAP25 silencing efficiency in C91-PL cells. (A) Flow cytometry analysis of C91-PL transduced cells with shSNAP25_1 (green) or shSNAP25_2 (yellow) or shSNAP25_All (blue turquoise), were stained

with an anti-SNAP25 antibody. C91-PL wild type (WT) cells not stained were used as negative control (dark grey, light grey and lime green) and C91-PL WT cells stained with the anti-Caveolin-1 antibody were used as the positive control (red, violet and orange). Cells were analyzed day 7 (D7) and day 14 (D14) after transduction. Transduction was repeated fpr the combination shSNAP25_All. Results are normalized. **(B)** Western blot analysis of C91-PL cells transduced with shSNAP25_1 or shSNAP25_2 or shSNAP25_All at D11 (left). C91-PL transduced for the second time with shSNAP25_All was analyzed at D14 (middle) C91-PL WT cells were used as positive control. Band's intensity was analyzed by ImageJ software and the rate of SNAP25 *per* Actin was represented in a bar graph (right). **(C)** Representative graph of cell's viability over time of C91-PL transduced cells with shSNAP25_1 (dark blue line) or shSNAP25_2 (yellow) or shSNAP25_All (first transduction, dark green line) and shSNAP25_All (second transduction, light green). C91-PL cells were also transduced with LV_GFP (control of transduction, dark blue line). Positive control was C91-PL WT cells (brown line). Range of days were cells have high viability (red area) are represented and range where silencing, and cell's viability is optimal is restricted by the vertical lines (dashed lines).

Besides the study of the role of Caveolin-1, we also studied the importance of SNAP25 protein during HTLV-1 replicative cycle and transmission. For this, we used shRNA delivery using HIV-derived lentivectors. The same experimental procedure was used to produce shRNA lentivector targeting SNAP25 expression. C91-PL cells were transduced with 3 or 4 lentivectors (MOI=1 for each lentivector) corresponding to three combinations. The first combination, shSNAP25_1, required three lentivectors, as well as the second combination, shSNAP25_2. A third combination, shSNAP25_All, involved the use of 4 lentivectors. As a control, C91-PL cells were transduced with LV-GFP.

Cells were first harvested 7 days after transduction. Since a significant amount of cells died within the first days after transduction, similarly to what observed for Caveolin-1 silencing. Silencing of SNAP25, was analyzed D7 and D14 by flow cytometry (**Figure 17A**). At D7, shSNAP25_1 combination seemed to be the most efficient with significant silencing of the SNAP25 protein. However, at D14 the combination shSNAP25_All lead to a more pronounced decrease of SNAP25 expression, revealing itself as better than shSNAP25_1. On the other hand, shSNAP25_2 was also more efficient at D14, but was overall less efficient than shSNAP25_1. These results were then confirmed by Western blot analysis performed on the same cells at D11 (**Figure 17B, left, D11**). All shRNA combination resulted in almost complete loss of SNAP25 expression. Signal intensity quantification reveals that silencing with shSNAP25_All combination was the most efficient. Thus, we repeated the transduction of C91-PL cells using only the combination shSNAP25_All and LV-GFP, as a control of transduction. Strikingly, this second transduction showed a different kinetic of silencing that did not fully recapitulate the first one. Indeed, the level of SNAP25 was lower at D7 than that of at D14 in the second experiment, while it was lower at D14 than at D7 in the first one **(Figure 17A, compare the two experiments**). This could be due to different titer of lentivector preparation, allowing better silencing efficacy when higher. Unfortunately, technical issues compromised evaluation of titers for the second experiments. Silencing was also confirmed western blot analysis (**Figure 17B, D14**). The viability of the cells in the first days after transduction decreases significantly, probably, because this SNAP25 protein is important for the cells (**Figure 17C**). In this way, the complete silencing promotes cell death. Over time, cell viability is normalized, and this corresponds exactly to the period during which SNAP25 silencing is almost complete (**Figure 17C, red area**). Thus, it is possible that the cells develop a compensatory mechanism, allowing them to survive without the SNAP25 protein.

Nonetheless, cells were left in culture until D30 after transduction. During this time, cell's viability started to decrease (**Figure 17C**), and after flow cytometry and western blot analysis, we observed that these cells expressed SNAP25 protein again (**Figure *18***), further confirming the reversion hypothesis.

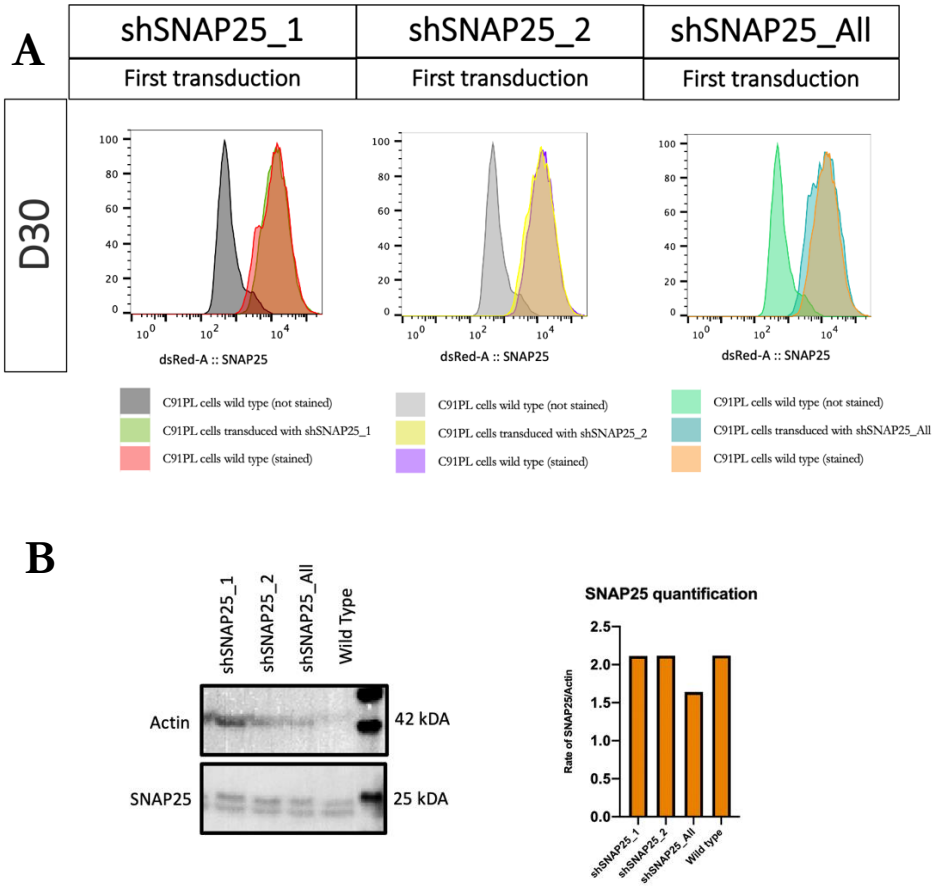
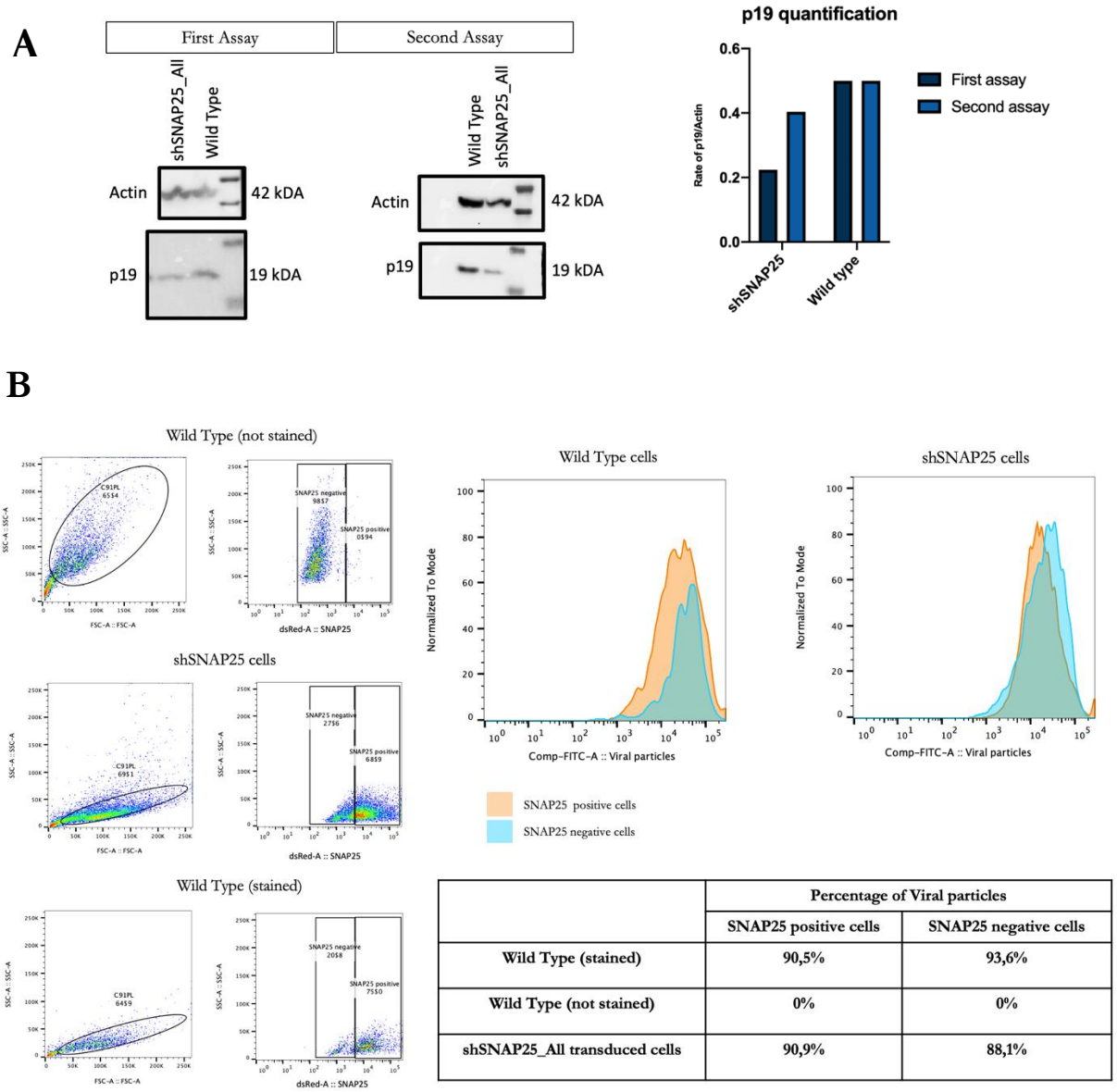


Figure 18 - SNAP25 silencing after 30 days of transduction. (A) Flow cytometry analysis of C91-PL transduced cells with shCaveolin-1_1 and stained with an anti-Caveolin-1 antibody (red). C91-PL wild type (WT) cells not stained were used as negative control (orange) and C91-PL WT cells stained with the anti-Caveolin-1 antibody were used as the positive control (blue). Cells were analyzed day 30 (D30) after transduction. Results are normalized. **(B)** Western blot analysis (left) of C91-PL cells transduced with shCaveolin-1_1, at D30. C91-PL WT cells were used as positive control. Band's intensity was analyzed by ImageJ software and the rate of Caveoli-1 *per* Actin was represented in a bar graph (right).

It is likely that transduced cells died, allowing the growth of the non-transduced cells, delayed in time due to their very low amount. Curiously, Western blot revealed a second bands of lower molecular weight than SNAP25 (**Figure 18B, left**). Some studies have already shown that another protein of the SNARE complex, the SNAP23, could be important for infected cells. Thus, it is possible that silencing of SNAP25 induced compensatory mechanism leading to expression of other SNAP. This needs to be confirmed using a specific SNAP23 antibody.

Altogether, these results defined that the best silencing is obtained using the shSNAP25_All combination, analyzed D11 and D14 after transduction (**Figure 17C, dashed lines**), in which cell viability is close to normal.

5.4. Viral expression in SNAP25 silenced cells



	Percentage of Viral particles	
	SNAP25 positive cells	SNAP25 negative cells
Wild Type (stained)	90,5%	93,6%
Wild Type (not stained)	0%	0%
shSNAP25_All transduced cells	90,9%	88,1%

Figure 19 - Evaluating of viral expression in SNAP25 silenced cells. (A) Western blot analysis (left) of C91-PL cells transduced with shSNAP25 at different timepoints. Membranes were stained with p19 antibody followed by actin antibody. C91-PL WT cells were used as positive control. Band's intensity was analyzed by ImageJ software and the rate of p19 per Actin was represented in a bar graph (right). **(B)** Flow cytometry analysis of C91-PL transduced cells with shSNAP25. Cells were first stained with human serum HTLV, permeabilized and stained why anti-SNAP25 antibody. C91-PL wild type (WT) cells not stained were used as negative control and C91-PL WT cells stained with the anti-SNAP25 antibody were used as the positive control. Plots with the chosen gating's are presented (left) for all the conditions regarding the expression of SNAP25. Cells with positive expression of SNAP25 (orange) and with negative expression of SNAP25 (blue) are represented in the histograms (top right) regarding the quantity of viral particles present in each condition. Results are normalized and simplified in a table (bottom right).

We next evaluated the production of viral particles in SNAP25 silenced C91-PL cells. First, we determined the level of p19 Gag by western blot 14 days after transduction, in independent experiments. Membranes were labelled with an antibody against the p19, a protein that belongs to the MA of HTLV-1 particles. In contrast to what was observed after Caveolin-1 silencing, knockdown of the SNAP25 protein decreased the level of p19 normalized to the level of Actin ratio

compared to that of WT condition (**Figure 19A, left and right).** Quantification of band intensity normalized to that of actin in each samples showed a decreased of 55-19 % of p19 expression in SNAP25 silenced cells. The difference of p19 expression was correlated with the efficiency of silencing: for the first assay, silencing was 95 % while for the second it was 80% as determined in (**Figure 19B, left).** So, in the second independent experiment, we observed that the decrease in p19 expression was less pronounced. This could be explained, since cells already had a higher expression of SNAP25 protein than the first assay (**Figure 17A, second essay, D14**), so the values were getting closer to the values of WT cells. Viral expression was also measured at the surface using human HTLV serum without cell permeabilization (**Figure 19B).** Strikingly, SNAP25 expression was almost similar in WT and shSNAP25 transduced cells, and viral expression was not different in transduced cells compared to WT. Furthermore, at the same timepoint, cells were analyzed by immunofluorescence and confocal microscopy (data not shown) and as the silencing was almost lost, it was not possible to observe differences between the control conditions and the shSNAP25 cells.

5.5. Viral transmission in SNAP25 silenced cells

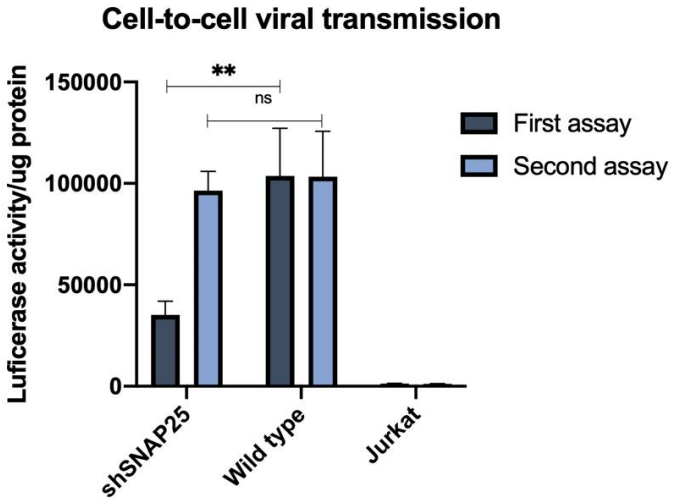


Figure 20 - Evaluation of cell-to-cell viral transmission in SNAP25 silenced cells. C91-PL wild type cell, shSNAP25 cells and Jurkat cells were co-cultured with Jurkat LTR-luc cells. Viral transmission is proportional to the quantity of Luciferase protein produced by Jurkat LTR-luc cells. Luciferase is quantified by fluorescence and results were presented in a bar graph.

After co-culture of shSNAP25, wild type and Jukat cells with Jurkat LTR-luc cells, luciferase protein was measured. There was a decrease in viral transmission by cell-to-cell contact when there is a reduction in SNAP25 protein expression (**Figure 20**). Furthermore, it seems that the lower the SNAP25 protein expression, less viral transmission is observed. As said before, in the second assay, cells expressed more SNAP25 than in the first assay and the viral transmission was higher for the second assay, approaching the values of viral transmission in wild type cells. As expected, Jurkat cells are not infected cell lines, so there is any viral transmission.

5.6. Evaluating silencing efficiency in MT-2 cells

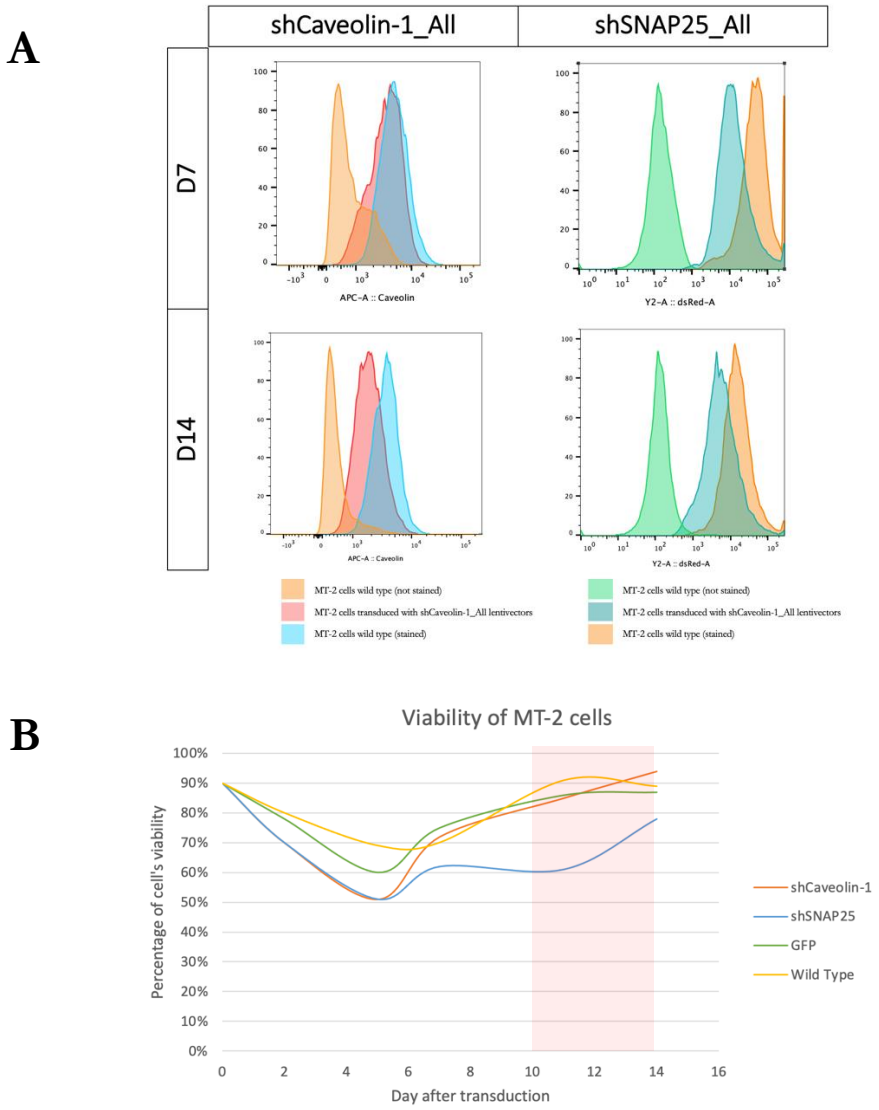


Figure 21 - Evaluating Caveolin-1 and SNAP25 silencing efficiency in MT-2 cells. (A) Flow cytometry analysis of MT-2 transduced cells with shCaveolin-1_All (red) or shSNAP25_All (blue turquoise). MT-2 wild type (WT) cells not stained were used as negative control (orange and green). MT-2 WT cells stained either with an anti-Caveolin-1 antibody (light blue) or with an anti-SNAP25 antibody (orange) were used as the positive control. Cells were analyzed day 7 (D7) and day 14 (D14) after transduction. Results are normalized. **(B)** Representative graph of cell's viability over time of MT-2 transduced cells with shCaveolin-1_All (orange line) or shSNAP25_All (blue line). MT-2 cells were also transduced with LV_GFP (control of transduction, green line). Positive control was MT-2 WT cells (yellow line). Range of days were cells have high viability (red area) are represented.

To confirm the effect of Caveolin-1 and SNAP25 on viral transmission and to avoid cell line effect, we used another HTLV-1 chronically infected cell line, the MT-2 cells. We repeated with MT-2 the same experiment using the combination and kinetics defined for the C91-PL cells. Thus, cells were traduced with the combination shCaveolin-1_All, and the combination shSNAP25_All using a MOI=1 for each lentivector used. Silencing was assessed by flow cytometry at D7 and D14 after transduction. At D7, SNAP25 protein expression was decreased, however that of Caveolin-1

was not (**Figure 21A**). At D14, a significant silencing was observed for both proteins, which led us to conclude that it would be the best period to perform the remaining analysis.

As for C91-PL, viability of MT-2 decreased in the first days after transduction (**Figure 21B**). However, shRNA transduction changed the behavior of MT-2. MT-2 cells are suspension cells, but after transduction they were like-adherent cells in the bottom of the multi-well plate, requiring a stronger pipetting to promote the detachment. Cells transduced with LV-GFP had, however, a similar behavior as the wild type. Thus, we can conclude that the damage of the cells is not solely due to the transduction, but probably because both Caveolin-1 and SNAP25 protein are important for cell survival. Therefore, probably the cells with a complete silencing died, and those that had partial silencing where able to survive. Hence, we observed that between D10 and D14 after transduction, the cells had good viability, and at the same time they had some silencing of either Caveolin-1 or SNAP25 (**Figure 21B, red area**).

5.7. Viral expression in MT-2 cells after Caveolin-1 silencing

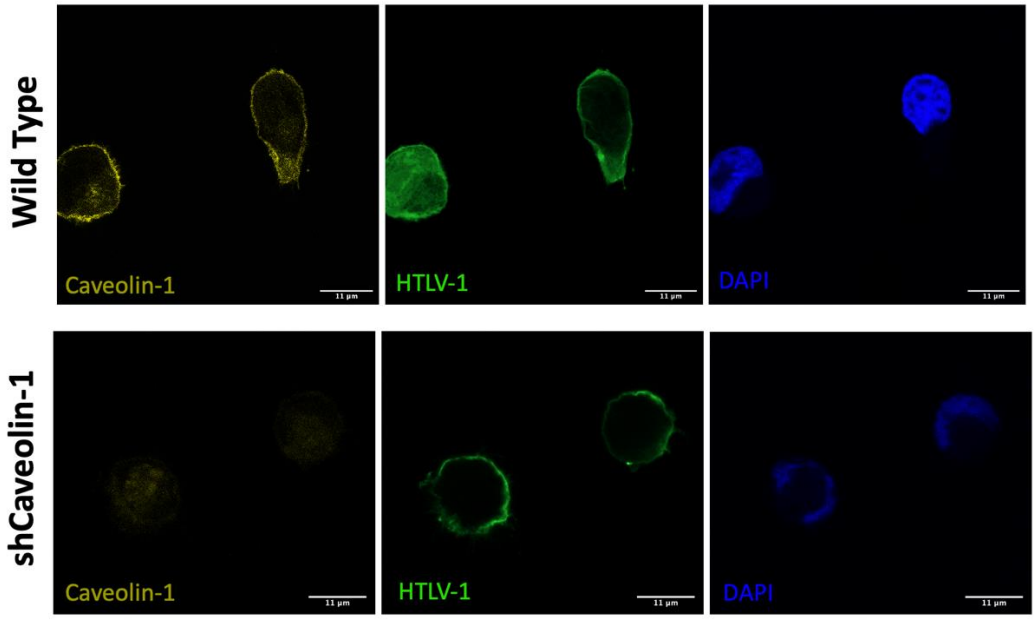


Figure 22 - Viral expression in MT-2 cells after Caveolin-1 silencing. Confocal microscopy was performed 14 days after transduction in MT-2 cells transduced with shCaveolin-1 (bottom line) and in wild type cells (top line). All cells were stained with anti-Caveolin-1 antibody (yellow), human serum HTLV-1 (green) and DAPI (blue). Scale=11μm.

Viral expression was simply observed for MT-2 shCaveolin-1 cells by confocal microscopy. Caveolin-1 expression was confirmed, neither viral expression, nor its density at the surface seemed different from that of non-transduced cells **(Figure 22)**. One of the wild type cells shown in the image seems to have a higher density of viral particles, but this is just a difference in planes. For this cell the captured plane is much closer to the cell surface when compared to the other cell. Hence, shCaveolin-1 cells do not present any alteration at the level of viral particle expression at the cell surface.

5.8. Viral transmission in MT-2 cells after silencing

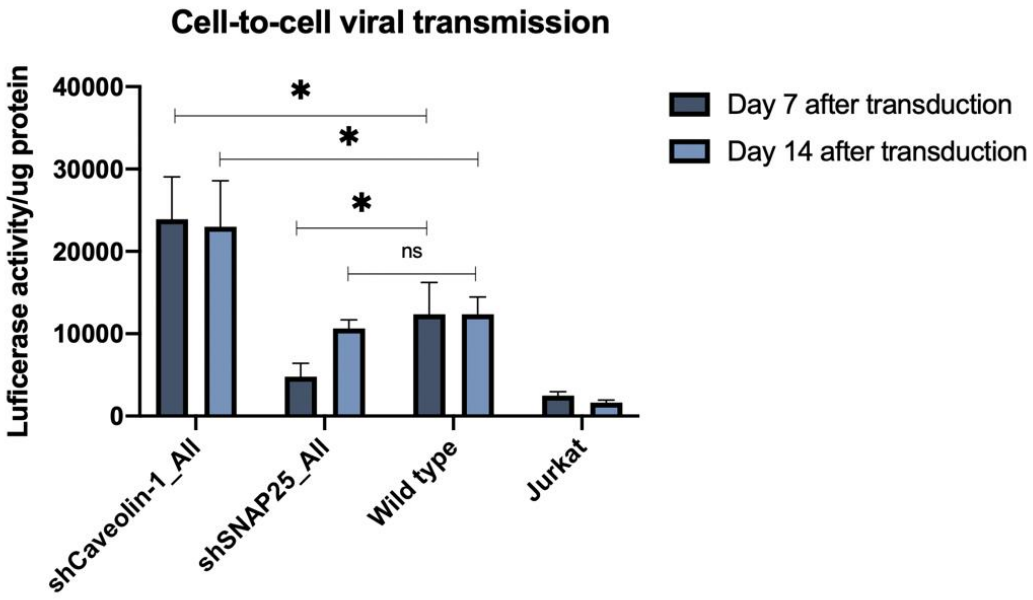


Figure 23 - Evaluation of cell-to-cell viral transmission in Caveolin-1 and SNAP25 silenced cells. MT-2 wild type cells, shCaveolin-1_All cells, shSNAP25_All cells and Jurkat cells were co-cultured with Jurkat LTR-luc cells. Viral transmission is proportional to the quantity of Luciferase protein produced by Jurkat LTR-luc cells. Luciferase is quantified by fluorescence and results were presented in a bar graph.

Viral transmission was measured by Luciferase assay after co-culture of WT MT-2, shCaveolin-1_All, shSNAP25_All and Jurkat cells with Jurkat LTR-luc cells. Silencing of Caveolin-1 lead to an almost two-fold increase in viral transmission compared with WT cells at the two time points (**Figure *23***). Curiously, Caveolin-1 expression at D7 was not reduced (**Figure 21A**). Transduction with control GFP-lentivector lead to viral transmission similar to that of non-transduced cells (supplementary data 2) suggesting that transduction by it-self did not change the ability of cells to transfer HTLV to target cells. However, we could not confirm Caveolin-1 silencing by western blot by sake of time, and we cannot exclude that the level of Caveolin-1 expression determined by FACS is not relevant to its amount that would be determined by western blot.

Silencing of SNAP25 was associated with a decrease in viral transmission in the two cell lines used (**Figure *23* and 20**) and consistent to its reduced expression evaluated by flow cytometry (**Figure 19**).

Chapter 6

Discussion

HTLV-1 is transmitted mainly through cell-to-cell contact since its free viral particles are poorly infectious. Given the importance of cell-to-cell contact for HTLV-1, many studies have been done in this domain. It is known that besides the two forms of cell-to-cell transmission, virological synapse, and cellular conduits, described for some viruses, HTLV-1 presents a unique form of viral transmission, called viral biofilm, in which viral particles are retained at the cell's surface until contact with other cells. The mechanisms and proteins involved in the process of viral biofilm biogenesis and maintenance are still not clear.

In this work, we evaluated the importance and the role of two membrane proteins for the HTLV-1 biofilm biogenesis and viral transmission. We performed several independent silencing experiments using two cell lines and showed that while Caveolin-1 silencing increased both p19 expression and viral transmission it did not change the level of virus at the surface of infected cells. In contrast, SNAP 25 silencing decreased viral transmission as well as p19 cytoplasmic level but not the surface viral protein expression nor biofilm distribution.

6.1. Viral biofilm transmission does not require Caveolin-1

Caveolin-1 is a protein known to be involved in endocytosis and exocytosis processes. After silencing of the protein, HTLV-1 infected cells behave differently in culture compared with the wild type cells. Furthermore, cell's viability is greatly affected. However, this does not seem to be associated to transduction, as cells transduced with a lentivector for GFP protein upregulation did not have their behavior altered. Thus, it seems that reduction of the Caveolin-1 protein, promotes high damage, and great mortality of the cells in culture. After one week, it was observed an efficient silencing of Caveolin-1 and at the same time re-establishment of the normal values of cell's viability. Therefore, it suggests that Caveolin-1 is important for cell survival, and that total silencing of this protein promotes cell death, so only cells with partially decreased Caveolin-1 expression are able to remain in culture with good viability. Furthermore, it seems that cells with low Caveolin-1 expression have a shorter lifespan, since cells highly silenced dyed in the first days and the silencing was lost over time. In other words, it is possible that the cells re-stablish the expression the Caveolin-1, or that cells with silencing die, while those not silenced survive and grow. This second hypothesis seems to be more reliable, since a few days after describing an efficient silencing, cell viability decreased again, and after that Caveolin-1 protein expression had been restored. At the end, we observe only not modified cells.

Nonetheless, the silencing of Caveolin-1 in infected cells promoted an increase of p19 inside the cells, however the density of viral particles at the cell surface was not changed. With these results, it is possible to infer that the Caveolin-1 protein is not involved in the maintenance of the viral biofilm, neither in the retention of the viral particles at the surface. However, the expression of p19 in the cytoplasm was increased. As Caveolin-1 is involved in exocytosis processes, it is possible that silencing of this protein reduces virus exocytosis, promoting its retention in the intracellular compartment. Thus, we expected that viral transmission would be decreased, however it was

observed that viral transmission increased in cells with silencing of Caveolin-1 relative to wild type cells. These surprising results were observed both in the C91-PL cell line and in the MT-2 cells. The proposed hypothesis, then, is that knock-down of Caveolin-1 promotes viral accumulation inside the cells, and that after cell-to-cell contact there is viral transmission via virological synapse, cellular conduits or even another yet unknown way of cell-to-cell transmission. To confirm this hypothesis, the supernatant of cells with reduced Caveolin-1 expression was collected and will be analyzed by ELISA. This new assay will allow the quantification of p19 in the extracellular medium of the modified cells compared to wild type ones.

6.2. SNAP25 is important for cell-to-cell transmission

In addition to the study of Caveolin-1, we also wanted to study the importance of the SNAP25 protein in HTLV-1 cell-to-cell transmission. Using the same silencing strategy based on shRNA transporter lentivectors we were able to observe viral transmission in the absence of SNAP25 protein.

Firstly, and once again, the knock-down promoted a decrease in cell viability and the number of cells in culture was severely affected during the first days. To achieve efficient silencing of SNAP25 protein, three combinations of shRNA's were tested. In the first cell analysis, the number of cells in culture was higher for the condition that promoted the lowest level of silencing. In addition, cells transduced with a lentivector designed to promote GFP protein expression were not as damaged as cells transduced with a shRNA. Indeed, LV-GFP transduced cells behaved very similarly in culture to wild type cells. Thus, it would be intuitive to conclude that this is not due to the transduction *per se*, but to the expression levels of SNAP25. Probably this protein is important for cell survival. Although this seems to be true, I believe that this protein is not as indispensable as Caveolin-1, or, cells have arranged a compensatory mechanism for the lack of SNAP25. It is possible to weave this conclusion, since at some timepoint the cells had an almost total silencing of the SNAP25 protein and at the same time normal values of cell viability, something that was not observed during Caveolin-1 silencing.

The knock-down of SNAP25 protein, promoted a decrease of p19 protein present in the cells, i.e. it seems that contrary to the action of Caveolin-1, SNAP25 silencing does not promote the retention of viral particles inside the cells. Thus, it is possible that SNAP25 is involved in the synthesis or building of viral particles, and that the suppression of this protein leads to a decrease in the amount of virions. Another hypothesis is that SNAP25 is involved in particle retention or inhibit budding of the viral particles and when this protein is suppressed the particles are released from the cells. Next, we want to assess the amount of viral particles on the surface, but unfortunately the cells had already restored the majority of SNAP25 protein expression. So, although it seems that the number of surface virions also decreased, the results are not very significant, since silencing was already greatly reduced. However, the supernatant of the cells during efficient silencing was harvest

and the number of released viral particles from the extracellular medium will be analyzed by ELISA. This will help us to understand if the SNAP25 protein is important during viral particle synthesis or if it is involved in the retention of virions.

In addition, knock-down of SNAP25 protein was also found to promote a decrease in viral transmission via cell contact. Once more, this suggests that SNAP25 silencing promotes either the release of viral particles that are weakly infectious or that the production of virions is reduced. Analysis by ELISA will be important for a better understanding of the role of SNAP25 protein. The SNAP25 protein belongs to the SNARE complex, which is responsible for the delivery of vesicular cargo, by promoting the fusion between vesicles and target cell membranes. As already demonstrated, the STX4 protein of the SNARE complex contributes to HIV-1 viral dissemination[110]. Thus, it is possible that SNAP25 has a similar effect during HTLV-1 viral transmission.

Finally, we can conclude that Caveolin-1 does not play a vital role in the maintenance of the viral biofilm. As for SNAP25, it may be that this protein promotes a decrease in viral biofilm, and therefore a decrease in transmission. However, the results did not allow us to draw reliable conclusions about the role or importance of the SNAP25 protein in the viral biofilm. Anyway, we can conclude that both Caveolin-1 and SNAP25 proteins play an important role in cell viability and in HTLV-1 transmission.

ix. Conclusion and future perspectives

HTLV-1 is almost exclusively transmitted by cell-to-cell contact. Currently, it is known that this virus has, besides the virological synapse and cellular conducts, a transmission route called viral biofilm. The biofilm is an extracellular matrix highly rich in adhesive molecules, water, and proteins. This type of structure is common in bacteria. However, its association to viruses is recent and still generates some disagreement between the scientific community. Until now, it is known that the only virus that has this biofilm-like structure is the HTLV-1. However, unpublished works have demonstrated that HIV-1 is also associated to a biofilm. Something that is not surprising given all the similarities between these two retroviruses. Interestingly, the big scientific development in the last year regarding SARS-Cov-2 has also hypothesized that this virus is associated with a viral biofilm.

The viral biofilm is responsible for the retention of viral particles on the surface of cells. This may be important in increasing viral density at the surface and the likelihood of a viral particle coming into contact with a target cell, which could explain why viral particles are polarized in infected cells. Furthermore, this structure could serve as a form of protection against antiviral treatments and the immune system. Doubts like these and many others still need to be clarified.

Currently, it is known that HTLV-1 biofilm is composed by Galactin-1, Tetherin, Colagen IV, Agrin among many other proteins. However, their importance and role are not clear. In this work, we direct our attention to the study of the importance and role of Caveolin-1 and SNAP25 protein at viral transmission and viral biofilm biogenesis. Caveolin-1 protein is involved in viral endocytosis processes, for example of HIV-1, moreover it had its expression increased in HTLV-1 infected cells. Therefore, the study of this protein seemed to be important to better understand the viral transmission. Thus, we observed, that this protein is either responsible for restricting the expression/production of viral particles or it promotes their release from the cells. Anyway, it does not seem that the release is made towards the biofilm. It was found that the knockdown of this protein does not promote changes in the density of viral particles in the biofilm. Therefore, Caveolin-1 seems to restrict viral transmission. Anyway, more results need to be obtained to understand the role of this protein.

The other protein we studied was SNAP25, a protein that belongs to the SNARE complex which is known to transport particles and vesicles between cells. Contrary to the results obtained for the Caveolin-1 protein, the SNAP25 protein is actually responsible for increasing viral transmission. And it seems to have some influence on the density of particles in the biofilm. However, the results are not significant enough to draw a firm conclusion about the importance of this protein in the viral biofilm. For both proteins, the density of cell-free viral particles in the cell supernatant is being analyzed, which will be important to understand the localization of the proteins.

The discovery of the functions of these proteins for viral transmission could be used to construct antiviral treatments, which could target both the biofilm and the proteins that compose this structure. Finally, the discoveries made may serve as a template to study the function of viral biofilm in other viruses.

x. Bibliography

1. Mahieux, R. and A. Gessain, *HTLV-3/STLV-3 and HTLV-4 viruses: discovery, epidemiology, serology and molecular aspects.* Viruses, 2011. **3**(7): p. 1074-90.
2. Latinne, A., et al., *Origin and cross-species transmission of bat coronaviruses in China.* Nature Communications, 2020. **11**(1): p. 4235.
3. Gessain, A., et al., *HTLV-3/4 and simian foamy retroviruses in humans: discovery, epidemiology, cross-species transmission and molecular virology.* Virology, 2013. **435**(1): p. 187-99.
4. Al Mussaed, E., *Human T-Cell Leukemia Lymphoma Virus (HTLV): From Discovery to Oncogenesis.* Cancer Biology 2015;5(1):1-10]. 2015. **55**: p. 1-10.
5. Gessain, A. and O. Cassar, *Epidemiological Aspects and World Distribution of HTLV-1 Infection.* Front Microbiol, 2012. **3**: p. 388.
6. Melamed, A., et al., *Clonality of HTLV-2 in natural infection.* PLoS Pathog, 2014. **10**(3): p. e1004006.
7. Eusebio-Ponce, E., et al., *HTLV-1 infection: An emerging risk. Pathogenesis, epidemiology, diagnosis and associated diseases.* Rev Esp Quimioter, 2019. **32**(6): p. 485-496.
8. Futsch, N., R. Mahieux, and H. Dutartre, *HTLV-1, the Other Pathogenic Yet Neglected Human Retrovirus: From Transmission to Therapeutic Treatment.* Viruses, 2017. **10**(1).
9. Nagasaka, M., et al., *Mortality and risk of progression to adult T cell leukemia/lymphoma in HTLV-1-associated myelopathy/tropical spastic paraparesis.* Proc Natl Acad Sci U S A, 2020. **117**(21): p. 11685-11691.
10. Mehta-Shah, N., L. Ratner, and S.M. Horwitz, *Adult T-Cell Leukemia/Lymphoma.* J Oncol Pract, 2017. **13**(8): p. 487-492.
11. Nagasaka, M., et al., *Mortality and risk of progression to adult T cell leukemia/lymphoma in HTLV-1–associated myelopathy/tropical spastic paraparesis.* Proceedings of the National Academy of Sciences, 2020. **117**: p. 11685-11691.
12. Bangham, C.R.M., *Human T Cell Leukemia Virus Type 1: Persistence and Pathogenesis.* Annu Rev Immunol, 2018. **36**: p. 43-71.
13. Nosaka, K. and M. Matsuoka, *Adult T-cell leukemia-lymphoma as a viral disease: Subtypes based on viral aspects.* Cancer Science, 2021. **112**(5): p. 1688-1694.
14. Chandhasin, C., et al., *Human T-cell leukemia virus type 1 tax attenuates the ATM-mediated cellular DNA damage response.* J Virol, 2008. **82**(14): p. 6952-61.
15. Akbarin, M.M., et al., *Evaluation of the role of TAX, HBZ, and HTLV-1 proviral load on the survival of ATLL patients.* Blood Res, 2017. **52**(2): p. 106-111.
16. Shimauchi, T., K. Kabashima, and Y. Tokura, *Adult T-cell leukemia/lymphoma cells from blood and skin tumors express cytotoxic T lymphocyte-associated antigen-4 and Foxp3 but lack suppressor activity toward autologous CD8+ T cells.* Cancer Sci, 2008. **99**(1): p. 98-106.
17. Bangham, C.R., et al., *HTLV-1-associated myelopathy/tropical spastic paraparesis.* Nat Rev Dis Primers, 2015. **1**: p. 15012.
18. Umekita, K. and A. Okayama, *HTLV-1 Infection and Rheumatic Diseases.* Frontiers in Microbiology, 2020. **11**(152).
19. Ramos, J.-M., C. de Mendoza, and V. Soriano, *HTLV-1 infection and health outcomes.* The Lancet Infectious Diseases, 2020. **20**(4): p. 407-408.
20. Kamoi, K. and M. Mochizuki, *HTLV-1 uveitis.* Front Microbiol, 2012. **3**: p. 270.
21. Kamoi, K., *HTLV-1 in Ophthalmology.* Front Microbiol, 2020. **11**: p. 388.
22. Rathsam-Pinheiro, R.H., et al., *Ocular lesions in HTLV-1 infected patients from Salvador, State of Bahia: the city with the highest prevalence of this infection in Brazil.* Revista da Sociedade Brasileira de Medicina Tropical, 2009. **42**: p. 633-637.
23. Matutes, E., et al., *Interferon alpha and zidovudine therapy in adult T-cell leukaemia lymphoma: response and outcome in 15 patients.* Br J Haematol, 2001. **113**(3): p. 779-84.

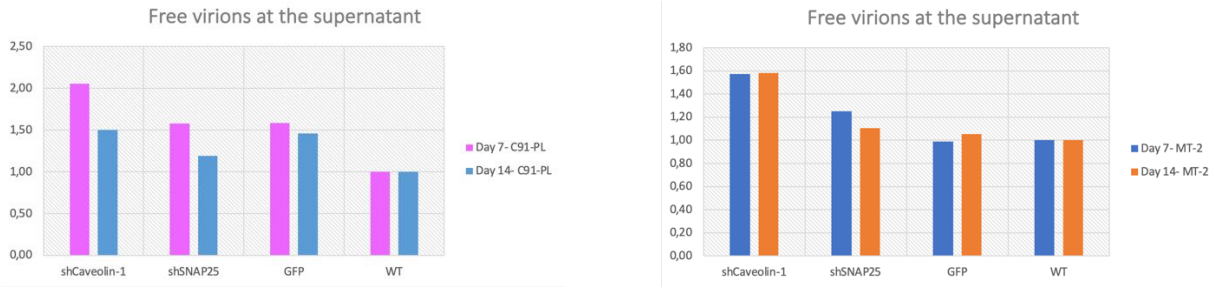
24. Kchour, G., et al., *Zidovudine and interferon-alpha treatment induces a high response rate and reduces HTLV-1 proviral load and VEGF plasma levels in patients with adult T-cell leukemia from North East Iran.* Leuk Lymphoma, 2007. **48**(2): p. 330-6.
25. Kinpara, S., et al., *Interferon-a (IFN-a) suppresses HTLV-1 gene expression and cell cycling, while IFN-a combined with zidovudine induces p53 signaling and apoptosis in HTLV-1-infected cells.* Retrovirology, 2013. **10**: p. 52.
26. Ishida, T., et al., *The CCR4 as a novel-specific molecular target for immunotherapy in Hodgkin lymphoma.* Leukemia, 2006. **20**(12): p. 2162-2168.
27. Taylor, G.P., et al., *Zidovudine plus lamivudine in Human T-Lymphotropic Virus type-I-associated myelopathy: a randomised trial.* Retrovirology, 2006. **3**(1): p. 63.
28. Boostani, R., et al., *Triple Therapy with Prednisolone, Pegylated Interferon and Sodium Valproate Improves Clinical Outcome and Reduces Human T-Cell Leukemia Virus Type 1 (HTLV-1) Proviral Load, Tax and HBZ mRNA Expression in Patients with HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis.* Neurotherapeutics, 2015. **12**(4): p. 887-95.
29. Olindo, S., et al., *Safety of long-term treatment of HAM/TSP patients with valproic acid.* Blood, 2011. **118**(24): p. 6306-9.
30. Zhang, L.L., et al., *Human T-cell lymphotropic virus type 1 and its oncogenesis.* Acta Pharmacol Sin, 2017. **38**(8): p. 1093-1103.
31. Ahmadi Ghezeldasht, S., et al., *Human T Lymphotropic Virus Type I (HTLV-I) Oncogenesis: Molecular Aspects of Virus and Host Interactions in Pathogenesis of Adult T cell Leukemia/Lymphoma (ATL).* Iranian journal of basic medical sciences, 2013. **16**: p. 179-195.
32. Catalan-Soares, B., A.B. Carneiro-Proietti, and F.A. Proietti, *Heterogeneous geographic distribution of human T-cell lymphotropic viruses I and II (HTLV-I/II): serological screening prevalence rates in blood donors from large urban areas in Brazil.* Cad Saude Publica, 2005. **21**(3): p. 926-31.
33. Garcia, I.F.d.S. and É.A. Hennington, *HTLV: uma infecção estigmatizante?* Cadernos de Saúde Pública, 2019. **35**.
34. Martin, F., Y. Tagaya, and R. Gallo, *Time to eradicate HTLV-1: an open letter to WHO.* Lancet, 2018. **391**(10133): p. 1893-1894.
35. Rosadas, C. and G.P. Taylor, *Mother-to-Child HTLV-1 Transmission: Unmet Research Needs.* Front Microbiol, 2019. **10**: p. 999.
36. Alais, S., R. Mahieux, and H. Dutartre, *Viral Source-Independent High Susceptibility of Dendritic Cells to Human T-Cell Leukemia Virus Type 1 Infection Compared to That of T Lymphocytes.* J Virol, 2015. **89**(20): p. 10580-90.
37. Jolly, C., et al., *HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse.* J Exp Med, 2004. **199**(2): p. 283-93.
38. Jolly, C. and Q.J. Sattentau, *Retroviral spread by induction of virological synapses.* Traffic, 2004. **5**(9): p. 643-50.
39. Hoshino, H., *Cellular Factors Involved in HTLV-1 Entry and Pathogenicit.* Front Microbiol, 2012. **3**: p. 222.
40. Gillet, N., et al., *Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human.* Retrovirology, 2007. **4**: p. 18.
41. Martin, J.L., et al., *Critical Role of the Human T-Cell Leukemia Virus Type 1 Capsid N-Terminal Domain for Gag-Gag Interactions and Virus Particle Assembly.* J Virol, 2018. **92**(14).
42. Mitchell, M.S., et al., *Synthesis, processing, and composition of the virion-associated HTLV-1 reverse transcriptase.* J Biol Chem, 2006. **281**(7): p. 3964-71.
43. Romanelli, M.G., et al., *Highlights on distinctive structural and functional properties of HTLV Tax proteins.* Front Microbiol, 2013. **4**: p. 271.
44. Martin, J.L., et al., *Molecular Studies of HTLV-1 Replication: An Update.* Viruses, 2016. **8**(2).
45. Maeda, Y., et al., *Separate cellular localizations of human T-lymphotropic virus 1 (HTLV-1) Env and glucose transporter type 1 (GLUT1) are required for HTLV-1 Env-mediated fusion and infection.* J Virol, 2015. **89**(1): p. 502-11.

46. Ghez, D., et al., *Neuropilin-1 is involved in human T-cell lymphotropic virus type 1 entry.* J Virol, 2006. **80**(14): p. 6844-54.
47. Baltimore, D., *RNA-dependent DNA polymerase in virions of RNA tumour viruses.* Nature, 1970. **226**(5252): p. 1209-11.
48. Rho, H.M., et al., *Characterization of the reverse transcriptase from a new retrovirus (HTLV) produced by a human cutaneous T-cell lymphoma cell line.* Virology, 1981. **112**(1): p. 355-360.
49. Niederer, H.A., et al., *HTLV-1 proviral integration sites differ between asymptomatic carriers and patients with HAM/TSP.* Virol J, 2014. **11**: p. 172.
50. Jones, K.S., et al., *Cell-free HTLV-1 infects dendritic cells leading to transmission and transformation of CD4(+) T cells.* Nat Med, 2008. **14**(4): p. 429-36.
51. Rizkallah, G., R. Mahieux, and H. Dutartre, *[Intercellular transmission of HTLV-1: not all mechanisms have been revealed].* Med Sci (Paris), 2015. **31**(6-7): p. 629-37.
52. Sze, A., et al., *Host restriction factor SAMHD1 limits human T cell leukemia virus type 1 infection of monocytes via STING-mediated apoptosis.* Cell Host Microbe, 2013. **14**(4): p. 422-34.
53. Ardeshna, et al., *Monocyte-derived dendritic cells do not proliferate and are not susceptible to retroviral transduction.* British Journal of Haematology, 2000. **108**(4): p. 817-824.
54. Kashanchi, F. and J.N. Brady, *Transcriptional and post-transcriptional gene regulation of HTLV-1.* Oncogene, 2005. **24**(39): p. 5938-51.
55. Miyazato, P., et al., *Transcriptional and Epigenetic Regulatory Mechanisms Affecting HTLV-1 Provirus.* Viruses, 2016. **8**(6).
56. Ching, Y.P., et al., *Specific TATAA and bZIP requirements suggest that HTLV-I Tax has transcriptional activity subsequent to the assembly of an initiation complex.* Retrovirology, 2004. **1**: p. 18.
57. Harrod, R., et al., *p300 and p300/cAMP-responsive element-binding protein associated factor interact with human T-cell lymphotropic virus type-1 Tax in a multi-histone acetyltransferase/activator-enhancer complex.* J Biol Chem, 2000. **275**(16): p. 11852-7.
58. Lefèbvre, L., et al., *Oncoviral bovine leukemia virus G4 and human T-cell leukemia virus type 1 p13(II) accessory proteins interact with farnesyl pyrophosphate synthetase.* J Virol, 2002. **76**(3): p. 1400-14.
59. Gazon, H., et al., *Human T-cell leukemia virus type 1 (HTLV-1) bZIP factor requires cellular transcription factor JunD to upregulate HTLV-1 antisense transcription from the 3' long terminal repeat.* J Virol, 2012. **86**(17): p. 9070-8.
60. Zhou, Q., T. Li, and D.H. Price, *RNA polymerase II elongation control.* Annu Rev Biochem, 2012. **81**: p. 119-43.
61. Geiger, T.R., et al., *The human T-cell leukemia virus type 1 tax protein confers CBP/p300 recruitment and transcriptional activation properties to phosphorylated CREB.* Mol Cell Biol, 2008. **28**(4): p. 1383-92.
62. Kulkarni, A., et al., *Glucose Metabolism and Oxygen Availability Govern Reactivation of the Latent Human Retrovirus HTLV-1.* Cell Chem Biol, 2017. **24**(11): p. 1377-1387.e3.
63. Hidaka, M., et al., *Post-transcriptional regulator (rex) of HTLV-1 initiates expression of viral structural proteins but suppresses expression of regulatory proteins.* Embo j, 1988. **7**(2): p. 519-23.
64. Toyoshima, H., et al., *Secondary structure of the human T-cell leukemia virus type 1 rex-responsive element is essential for rex regulation of RNA processing and transport of unspliced RNAs.* J Virol, 1990. **64**(6): p. 2825-32.
65. Rehberger, S., et al., *The activation domain of a hormone inducible HTLV-1 Rex protein determines colocalization with the nuclear pore.* Exp Cell Res, 1997. **233**(2): p. 363-71.
66. Fogarty, K.H., et al., *New insights into HTLV-1 particle structure, assembly, and Gag-Gag interactions in living cells.* Viruses, 2011. **3**(6): p. 770-93.
67. Maldonado, J.O., et al., *New insights into retroviral Gag-Gag and Gag-membrane interactions.* Front Microbiol, 2014. **5**: p. 302.

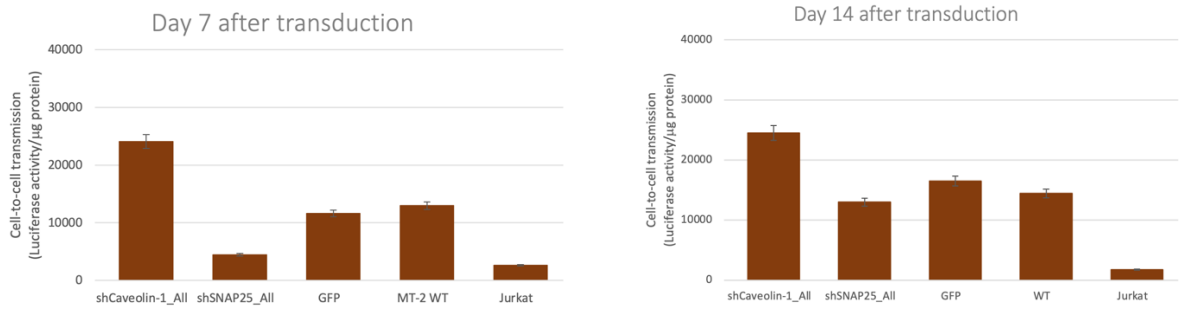
68. Olson, E.D. and K. Musier-Forsyth, *Retroviral Gag protein-RNA interactions: Implications for specific genomic RNA packaging and virion assembly.* Seminars in cell & developmental biology, 2019. **86**: p. 129-139.
69. Ceccaldi, P.E., et al., *DC-SIGN facilitates fusion of dendritic cells with human T-cell leukemia virus type 1-infected cells.* J Virol, 2006. **80**(10): p. 4771-80.
70. Rizkallah, G., et al., *Correction: Dendritic cell maturation, but not type I interferon exposure, restricts infection by HTLV-1, and viral transmission to T-cells.* PLoS Pathog, 2017. **13**(7): p. e1006494.
71. Slaughter, B. and R. Li, *Toward Quantitative "In Vivo Biochemistry" with Fluorescence Fluctuation Spectroscopy.* Molecular biology of the cell, 2010. **21**: p. 4306-11.
72. Konvalinka, J., H.G. Kräusslich, and B. Müller, *Retroviral proteases and their roles in virion maturation.* Virology, 2015. **479-480**: p. 403-17.
73. Jones, K.S., et al., *Human T-cell leukemia virus type 1 (HTLV-1) and HTLV-2 use different receptor complexes to enter T cells.* J Virol, 2006. **80**(17): p. 8291-302.
74. Nomaguchi, M., et al., *Viral Tropism.* Frontiers in Microbiology, 2012. **3**(281).
75. Jones, K.S., et al., *Heparan sulfate proteoglycans mediate attachment and entry of human T-cell leukemia virus type 1 virions into CD4+ T cells.* J Virol, 2005. **79**(20): p. 12692-702.
76. Manel, N., et al., *The ubiquitous glucose transporter GLUT-1 is a receptor for HTLV.* Cell, 2003. **115**(4): p. 449-59.
77. Gross, C. and A.K. Thoma-Kress, *Molecular Mechanisms of HTLV-1 Cell-to-Cell Transmission.* Viruses, 2016. **8**(3): p. 74.
78. Tang, F., Q. Du, and Y.-J. Liu, *Plasmacytoid dendritic cells in antiviral immunity and autoimmunity.* Science China Life Sciences, 2010. **53**(2): p. 172-182.
79. Rocamonde, B., et al., *HTLV-1 infection of myeloid cells: from transmission to immune alterations.* Retrovirology, 2019. **16**(1): p. 45.
80. Furuta, R., et al., *Human T-cell leukemia virus type 1 infects multiple lineage hematopoietic cells in vivo.* PLoS Pathog, 2017. **13**(11): p. e1006722.
81. de Castro-Amarante, M.F., et al., *The contribution of monocytes/macrophages to HTLV-1 infection and persistence.* Retrovirology, 2014. **11**(Suppl 1): p. P123-P123.
82. Alexaki, A. and B. Wigdahl, *HIV-1 infection of bone marrow hematopoietic progenitor cells and their role in trafficking and viral dissemination.* PLoS Pathog, 2008. **4**(12): p. e1000215.
83. Yasuma, K., et al., *HTLV-1 bZIP Factor Impairs Anti-viral Immunity by Inducing Co-inhibitory Molecule, T Cell Immunoglobulin and ITIM Domain (TIGIT).* PLoS Pathog, 2016. **12**(1): p. e1005372.
84. Carpentier, A., et al., *Modes of Human T Cell Leukemia Virus Type 1 Transmission, Replication and Persistence.* Viruses, 2015. **7**(7): p. 3603-24.
85. Carpentier, A., et al., *Modes of Human T Cell Leukemia Virus Type 1 Transmission, Replication and Persistence.* Viruses, 2015. **7**(7): p. 3603-3624.
86. Omsland, M., et al., *Inhibition of Tunneling Nanotube (TNT) Formation and Human T-cell Leukemia Virus Type 1 (HTLV-1) Transmission by Cytarabine.* Sci Rep, 2018. **8**(1): p. 11118.
87. Van Prooyen, N., et al., *Human T-cell leukemia virus type 1 p8 protein increases cellular conduits and virus transmission.* Proc Natl Acad Sci U S A, 2010. **107**(48): p. 20738-43.
88. Nejmeddine, M. and C.R. Bangham, *The HTLV-1 Virological Synapse.* Viruses, 2010. **2**(7): p. 1427-47.
89. Igakura, T., et al., *Spread of HTLV-I between lymphocytes by virus-induced polarization of the cytoskeleton.* Science, 2003. **299**(5613): p. 1713-6.
90. Theocharis, A.D., et al., *Extracellular matrix structure.* Advanced Drug Delivery Reviews, 2016. **97**: p. 4-27.
91. Maali, Y., et al., *Microbial Biofilms: Human T-cell Leukemia Virus Type 1 First in Line for Viral Biofilm but Far Behind Bacterial Biofilms.* Frontiers in Microbiology, 2020. **11**(2041).
92. Stoodley, P., et al., *Biofilms as complex differentiated communities.* Annu Rev Microbiol, 2002. **56**: p. 187-209.

93. Jefferson, K.K., *What drives bacteria to produce a biofilm?* FEMS Microbiology Letters, 2004. **236**(2): p. 163-173.
94. Pais-Correia, A.M., et al., *Biofilm-like extracellular viral assemblies mediate HTLV-1 cell-to-cell transmission at virological synapses.* Nat Med, 2010. **16**(1): p. 83-9.
95. Moser, C., et al., *Biofilms and host response - helpful or harmful.* Apmis, 2017. **125**(4): p. 320-338.
96. Assil, S., et al., *Sensing of cell-associated HTLV by plasmacytoid dendritic cells is regulated by dense β-galactoside glycosylation.* PLoS Pathog, 2019. **15**(2): p. e1007589.
97. Hojo, H., et al., *Total synthesis and structural characterization of caveolin-1.* Angewandte Chemie, 2021.
98. Sahay, B. and A. Mergia, *The Potential Contribution of Caveolin 1 to HIV Latent Infection.* Pathogens, 2020. **9**: p. 896.
99. Volonte, D. and F. Galbiati, *Caveolin-1, a master regulator of cellular senescence.* Cancer and Metastasis Reviews, 2020. **39**: p. 1-18.
100. Ni, K., et al., *The Evolving Role of Caveolin-1: A Critical Regulator of Extracellular Vesicles.* Medical Sciences, 2020. **8**: p. 46.
101. Tomassian, T., et al., *Caveolin-1 orchestrates TCR synaptic polarity, signal specificity, and function in CD8 T cells.* J Immunol, 2011. **187**(6): p. 2993-3002.
102. Lin, S., et al., *HIV Infection Upregulates Caveolin 1 Expression To Restrict Virus Production.* Journal of virology, 2010. **84**: p. 9487-96.
103. Lin, S., et al., *Caveolin-1 reduces HIV-1 infectivity by restoration of HIV Nef mediated impairment of cholesterol efflux by apoA-I.* Retrovirology, 2012. **9**: p. 85.
104. Sawada, S., et al., *Overexpression of caveolin-1 in adult T-cell leukemia.* Blood, 2010. **115**: p. 2220-30.
105. Sawada, S., et al., *Human T-Cell Leukemia Virus Type 1 Tax Activates Caveolin-1 Gene Expression.* Blood, 2008. **112**(11): p. 2816-2816.
106. Shaaban, A., et al., *The SNAP-25 linker supports fusion intermediates by local lipid interactions.* eLife, 2019. **8**: p. e41720.
107. Irfan, M., et al., *SNAP-25 isoforms differentially regulate synaptic transmission and long-term synaptic plasticity at central synapses.* Scientific Reports, 2019. **9**(1): p. 6403.
108. Machitani, M., et al., *MicroRNA miR-27 Inhibits Adenovirus Infection by Suppressing the Expression of SNAP25 and TXN2.* Journal of Virology, 2017. **91**(12): p. e00159-17.
109. Eletto, D., et al., *Inhibition of SNAP25 expression by HIV 1 Tat involves the activity of mi' 128a.* Journal of Cellular Physiology, 2008. **216**.
110. Jolly, C., et al., *The Regulated Secretory Pathway in CD4+ T cells Contributes to Human Immunodeficiency Virus Type-1 Cell-to-Cell Spread at the Virological Synapse.* PLOS Pathogens, 2011. **7**(9): p. e1002226.
111. Alcover, A. and M.I. Thoulouze, *Vesicle traffic to the immunological synapse: a multifunctional process targeted by lymphotropic viruses.* Curr Top Microbiol Immunol, 2010. **340**: p. 191-207.
112. Jain, P., et al., *Identification of human T cell leukemia virus type 1 tax amino acid signals and cellular factors involved in secretion of the viral oncoprotein.* J Biol Chem, 2007. **282**(47): p. 34581-93.

Supplementary data



S1 - Ratio of free viral particles present in the supernatant of infected cells after silencing. The supernatant of C91-PL and MT-2 wild type cells, shCaveolin-1_All cells, shSNAP25_All cells and cells transduced with LV-GFP were analyzed by ELISA. This assay was performed after the delivery of this manuscript. With these results we can see that there was an increase of viral particles in the supernatant of shCaveolin-1 cells. When we look for the shSNAP25 cells, it seems that there was no significative difference on the quantity of virions at the supernatant between cells and the MT-2 wilt type cells. However, for the C91-PL cells line, seems that the transduction by itself can increase the number of virions, but not the silencing of the protein SNAP25.



S2 - Cell-to-cell viral transmission in Caveolin-1 and SNAP25 silenced cells with the GFP control. MT-2 wild type cells, shCaveolin-1_All cells, shSNAP25_All cells, GFP-transduced cells and Jurkat cells were co-cultured with Jurkat LTR-luc cells. Viral transmission is proportional to the quantity of Luciferase protein produced by Jurkat LTR-luc cells. Luciferase is quantified by fluorescence and results were presented in a bar graph. GFP values are similar to the wild type ones.