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Identification of immunovirological factors that determine an extremely low viral reservoir: approaching the cure of HIV-1 infection

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2020

Thesis to obtain the PhD degree in
Biochemistry, Molecular Biology and Biomedicine of
the Universitat Autònoma de Barcelona

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Certifiquen:

Que el treball experimental realitzat i la redacció de la memòria de la Tesi Doctoral titulada: "**Identification of immunovirological factors that determine an extremely low viral reservoir: approaching the cure of HIV-1 infection**" han estat realitzades per la Cristina Gálvez Celada sota la seva direcció i consideren que és apte per a ser presentada per a optar al grau de Doctor en Bioquímica, Biologia Molecular i Biomedicina per la Universitat Autònoma de Barcelona.

I per tal que en quedi constància, signa aquest document a Bellaterra, 2 de Novembre del 2020.

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Dr. Francisco Rodríguez Frías

A mi familia y a Cristian,

"La ciencia nunca resuelve un problema sin crear otros 10 más"

- George Bernard Shaw

ABBREVIATIONS

1-LTR	1-Long Terminal Repeat
2-LTR	2-Long Terminal Repeat
ADCC	Antibody-Dependent Cellular Toxicity
AIDS	Acquired Immune Deficiency Syndrome
Allo-HSCT	Allogeneic Hematopoietic Stem Cell Transplant
ATG	AntiThymocyte Globulin
AZT	AZidoThymidine
BM	Bone Marrow
bNAbs	broadly Neutralizing Antibodies
CA	Capsid
caHIV-1 RNA	cell-associated HIV-1 RNA
cART	combined AntiRetroviral Therapy
CART	Classification And Regression Trees
CAR-T	Chimeric Antigen Receptors T cells
CCR5	CC Chemokine Receptor type 5
CD	Cluster of Differentiation
CDC	Centers for Disease Control
cp-LoViReT	Chronic phase - Low Viral Reservoir Treated
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/CRISP-Associated protein nuclease-9
CRP	C-Reactive Protein
CSF	CerebroSpinal Fluid
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
CXCR4	CXC Chemokine Receptor type 4

DAA	Direct Acting Antivirals
DCs	Dendritic Cells
ddPCR	droplet digital PCR
DNA	DeoxyriboNucleic Acid
dsDNA	double-stranded DNA
DTT	DiThioThreitol
dUTP	DeoxyUridine-5'-TriPhosphate
EC	Elite Controllers
EDTA	EthyleneDiamineTetraacetic Acid
EEC	Exceptional Elite Controllers
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMA	European Medicines Agency
ENV	Envelope
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
FISH	Fluorescence *In Situ* Hybridization
FNB	Fine Needle Biopsy
FPR	False Positive Rate
GAG	Group specific antigen
GALT	Gut Associated Lymphoid Tissue
GvHD	Graft-versus Host Disease
HAART	Highly Active Antiretroviral Therapy
HCV	Hepatitis C Virus
HD	Healthy Donors
HIC	HIV-1 Controllers

HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigens
HTLV-III	Human T-lymphotropic Virus type III
IFNα	Interferon α
IL	Interleukin
INIs	Integrase Inhibitors
IP10	Interferon gamma-induced Protein 10
IPDA	Intact Proviral DNA Assay
IQR	InterQuartile Range
IUPM	Infectious Unit Per Million cells
IVDU	IntraVenous Drug Use
LAV	Lymphadenopathy-Associated Virus
LN	Lymph Node
LOD	Limit Of Detection
LoViReT	Low Viral Reservoir Treated
LRA	Latency Reversal Agents
LTNP	Long-Term Non Progressors
LTR	Long Terminal Repeats
MA	Matrix
MMWR	Mortality and Mortality Weekly Reports
ms	multiple spliced
mVOA	murine Viral Outgrowth Assay
NC	NucleoCapsid
ND	Not Determined
NEF	Negative Factor

NK	Natural Killer
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTI	Nucleoside Reverse Transcriptase Inhibitor
PBMCs	Peripheral Blood Mononuclear Cells
PBPC	Peripheral Blood Progenitors Cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed cell Death protein 1
PHA	PhytoHemAgglutinin
PIC	Pre-Integration Complex
Pindex	Poly-functionality index
PIs	Protease Inhibitors
PMA	Phorbol 12-Myristate, 13-Acetate
POL	Polymerase
PS	Packaging Signal
PTC	Post-Treatment Controllers
pVL	plasma Viral Load
qPCR	quantitative PCR
qVOA	quantitative Viral Outgrowth Assay
RBV	Ribavirin
REV	Regulator of expression proteins
RNA	RiboNucleic Acid
RP	Rapid Progressors
RPMI	Rowell Park Memorial Institute
RPP30	Ribonuclease P/MRP Subunit P30

RRE	Rev-Responsive Element
rt-qPCR	reverse transcription quantitative PCR
SCA	Single Copy Assay
SIV	Simian Immunodeficiency Virus
SIMOA	SIngle-Molecule Array
SMAC	Second Mitochondria-derived Activator of Caspases
ssRNA	single-stranded RNA
STR	Short Tandem Repeats
TALEN	Transcription Activator-Like Effector Nucleases
TAT	Trans-activating regulatory protein
TBP	TATA-binding protein
TCFG	T-Cell Growth Factor
T$_{CM}$	T Central Memory
T$_{EF}$	T Effector Memory
T$_{EM}$	T Effector Memory
T$_{EMRA}$	T Effector Memory RA$^+$
T$_{FH}$	T Follicular Helper
TILDA	Tat/rev Induced Limiting Dilution Assay
T$_N$	T Naïve
TNF	Tumor Necrosis Factor
T$_{REG}$	T Regulatory
T$_{RM}$	Tissue Resident Memory
T$_{SCM}$	T Stem Cell Memory
T$_{TD}$	T Terminally Differentiated
T$_{TM}$	T Transitional Memory

UNAIDS	Joint United Nations Programme on HIV/AIDS
us	unspliced
usVL	ultrasensitive Viral Load
VC	Viremic Controllers
VIF	Virion Infectivity Factor
VNP	Viremic Non-Progressors
VPR	Viral Protein R
VPU	Viral Protein U
WHO	World Health Organization
ZFN	Zinc Fingers Nucleases
β2M	β2-Microglobulin

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SUMMARY, RESUMEN & RESUM

Nearly 40 million individuals are currently living with the human immunodeficiency virus type 1 (HIV-1) worldwide and more than 30 million deaths can be attributed to HIV-1 since the start of the epidemic. The development of combination antiretroviral therapy (cART) was one of the major medical success of the 20th century as it effectively suppresses plasma viremia, improves the immune system function, reduces mortality and morbidity, and the risk of viral transmission. However, cART cannot cure the infection due to the presence of latently infected cells.

The number of latently infected cells present in the body, known as the reservoir, varies notably among different HIV-1-infected individuals. Low levels of reservoirs are traditionally related to HIV-1 control or good disease prognosis, but the specific factors provoking a reduction of this reservoir are still unknown. In this context, the aim of this thesis is to characterize the clinical, viral and immunogenetic factors associated with the reduction of the HIV-1 reservoir in three different models: [1] a natural long-term control of the infection (Exceptional Elite controllers or EEC), [2] a natural low HIV-1 reservoir despite regular HIV-1 progression (Low Viral Reservoir Treated individuals or LoViReT), and [3] an induced low reservoir after allogeneic hematopoietic stem cell transplant (allo-HSCT) in persons living with HIV-1.

Our results showed that the low-level reservoirs observed in EEC were due to a combination of defective non-evolving virus with protective host genetic factors together with potent HIV-1 specific immune responses. Altogether, the combination of those factors seems important to control HIV-1 replication and reach a spontaneous functional cure. On the other hand, LoViReT individuals seem to have an impaired HIV-1 reservoir establishment since the low levels of reservoir were observed in blood (even before the initiation of treatment) and anatomical sanctuaries. This was supported by a skewed distribution of the provirus among the different CD4$^+$ T cells subpopulations towards short-live subpopulations. However, although LoViReT individuals have a preserved general immune system, they do not have HIV-1 specific immune responses and therefore are not able to control HIV-1 replication in the absence of cART. Finally, the study of HIV-1-infected individuals that received an allo-HSCT showed a remarkably decline of the HIV-1 reservoir in peripheral blood and anatomical compartments, even in the setting of

CCR5 wild-type cells. This was most likely due to a potent graft versus HIV-1 effect caused by specific factors of the transplant as a rapid engraftment, an adult donor, or a graft versus host disease. Unfortunately, the lack of a potent HIV-1 specific immune responses sustained in time would result in viral rebound, as observed in one of the studied individuals transplanted with *CCR5* wild-type cells.

Altogether, we showed that factors related to an impaired viral function will be important to the reduction of HIV-1 reservoir. A combination with a potent HIV-1 specific immune response will be necessary to control the HIV-1 replication in the absence of cART as happened in EEC. Future clinical trials in individuals with a low HIV-1 reservoir, like LoViReT or allo-HSCT individuals, would be of great interest in strategies aimed at boosting HIV-1 immune specific responses with the ultimate goal of curing HIV-1.

Casi 40 millones de personas viven actualmente con el virus de la inmunodeficiencia humana tipo 1 (VIH-1) en todo el mundo, y más de 30 millones de muertes se pueden atribuir al VIH-1 desde el comienzo de la epidemia. El desarrollo de la terapia antirretroviral combinada (TARc) es uno de los principales éxitos médicos del siglo XX, ya que suprime eficazmente la viremia plasmática, mejora la función del sistema inmunológico, reduce la mortalidad y morbilidad, y el riesgo de transmisión viral. Sin embargo, la TARc no puede curar la infección debido a la presencia de células infectadas de forma latente, lo que se conoce como reservorio viral.

El número de células latentemente infectadas presentes en el cuerpo varía notablemente entre diferentes individuos infectados por VIH-1. Bajos niveles de reservorios se han relacionado tradicionalmente con un control del VIH-1 o buen pronóstico de la enfermedad, pero los factores específicos que provocan esta reducción del reservorio aún se desconocen. En este contexto, el objetivo de esta tesis es caracterizar los factores clínicos, virales e inmunogenéticos asociados con la reducción del reservorio del VIH-1 en tres modelos diferentes: [1] controladores naturales de la infección con un largo seguimiento (Controladores de Elite Excepcionales o EEC), [2] individuos con un reservorio de VIH-1 espontáneamente bajo a pesar de una progresión normal de la infección (LoViReT), y [3] personas VIH-1^{+} en las que se observa una caída del reservorio después de un trasplante alogénico de células madre hematopoyéticas (allo-HSCT).

Nuestros resultados mostraron que los bajos niveles de reservorio observados en los EEC se debieron a la combinación de virus defectivos que no evolucionan con factores genéticos protectores del huésped junto con respuestas inmunitarias potentes específicas del VIH-1. En conjunto, la combinación de estos factores parece importante para controlar la replicación del VIH-1 y alcanzar una cura funcional espontánea. Por otro lado, los individuos LoViReT parecen tener un establecimiento defectivo del reservorio de VIH-1 ya que se observaron bajos niveles de reservorio en sangre (incluso antes del inicio del tratamiento) y santuarios anatómicos. Esto estaba acompañado por una distribución sesgada de la latencia en las diferentes subpoblaciones de células T CD4^{+} hacia subpoblaciones de vida más corta. Sin embargo, aunque los individuos LoViReT mostraron un sistema

inmunológico preservado, no se observaron respuestas inmunitarias específicas del VIH-1 y, por lo tanto, no se esperaría que controlaran la replicación del VIH-1 en ausencia de TARc. Finalmente, el estudio de individuos infectados por VIH-1 que recibieron un trasplante alogénico de células madre hematopoyéticas mostró una notable disminución del reservorio de VIH-1 en sangre periférica y compartimentos anatómicos, incluso en el contexto de células con un *CCR5* salvaje. Esto probablemente fue debido a un potente efecto de injerto contra VIH-1 causado por factores específicos del trasplante como un rápido quimerismo, células procedentes de un donante adulto y la presencia de enfermedad del injerto contra huésped. Desafortunadamente, la falta de respuestas potentes específicas de VIH-1 sostenidas en el tiempo resultaría en un rebote viral, como se observó en uno de los individuos estudiados que fue trasplantado con células con un *CCR5* salvaje.

En conjunto, demostramos que los factores relacionados con una función viral alterada son importantes para la reducción del reservorio de VIH-1. Aun así, para controlar la replicación del VIH-1 en ausencia de TARc será necesaria la combinación con respuestas inmunes potentes específicas de VIH-1, como sucede en los EEC. Futuros ensayos clínicos en personas con un reservorio bajo de VIH-1, como individuos LoViReT o que hayan recibido un trasplante alogénico de células madre, serían de gran interés en estrategias destinadas a estimular las respuestas inmunes específicas del VIH-1 con el objetivo final de curar el VIH-1.

Gairebé 40 milions de persones viuen actualment amb el virus de la immunodeficiència humana tipus 1 (VIH-1) a tot el món, i més de 30 milions de morts es poden atribuir al VIH-1 des del començament de l'epidèmia. El desenvolupament de la teràpia antiretroviral combinada (TARc) és un dels principals èxits mèdics del segle XX, ja que suprimeix eficaçment la virèmia plasmàtica, millora la funció del sistema immunològic, redueix la mortalitat i la morbiditat, i el risc de transmissió viral. No obstant, la TARc no pot curar la infecció a causa de la presència de cèl·lules infectades de manera latent, el que es coneix com a reservori viral.

El nombre de cèl·lules infectades latentment presents en el cos varia notablement entre diferents individus infectats pel VIH-1. Baixos nivells de reservoris s'han relacionat tradicionalment amb un control del VIH-1 o bon pronòstic de la malaltia, però els factors específics que provoquen aquesta reducció del reservori encara es desconeixen. En aquest context, l'objectiu d'aquesta tesi és caracteritzar els factors clínics, virals i immunogenètics associats a la reducció del reservori del VIH-1 en tres models diferents: [1] controladors naturals de la infecció amb un llarg seguiment (Controladors d'Elit Excepcionals o EEC), [2] individus amb un reservori de VIH-1 espontàniament baix malgrat una progressió normal de la infecció (LoViReT), i [3] persones amb VIH en les que s'observa una inducció de la caiguda del reservori després d'un trasplantament al·logènic de cèl·lules mare hematopoètiques (allo-HSCT).

Els nostres resultats van mostrar que els baixos nivells de reservori observats en els EEC es van deure a la combinació de virus defectius que no evolucionen amb factors genètics protectors de l'hoste juntament amb respostes immunitàries potents específiques del VIH-1. En conjunt, la combinació d'aquests factors sembla important per a controlar la replicació del VIH-1 i aconseguir una cura funcional espontània. D'altra banda, els individus LoViReT semblen tenir un establiment defectiu del reservori de VIH-1 ja que es van observar baixos nivells de reservori en sang (fins i tot abans de l'inici del tractament) i santuaris anatòmics. Això estava recolzat per una distribució esbiaixada de la latència en les diferents subpoblacions de cèl·lules T CD4$^+$ cap a subpoblacions de vida més curta. No obstant això, encara que els individus LoViReT van mostrar un sistema immunològic preservat, no es

van observar respostes immunitàries específiques del VIH-1 i, per tant, no s'esperaria que poguessin controlar la replicació del VIH-1 en absència de TARc. Finalment, l'estudi d'individus infectats per VIH-1 que van rebre un trasplantament al·logènic de cèl·lules mare hematopoètiques va mostrar una notable disminució del reservori de VIH-1 en sang perifèrica i compartiments anatòmics, fins i tot en el context de cèl·lules amb un *CCR5* salvatge. Això probablement va ser degut a un potent efecte de l'empelt contra VIH-1 causat per factors específics del transplantament com el ràpid quimerisme, cèl·lules procedents d'un donant adult i la malaltia de l'empelt contra l'hoste. Desafortunadament, la falta de respostes immunes potents específiques del VIH-1 sostingudes en el temps resultaria en un rebot viral, com es va observar en un dels individus estudiats trasplantat amb cèl·lules amb un *CCR5* salvatge.

En conjunt, demostrem que els factors relacionats amb una funció viral alterada seran importants per a la reducció del reservori del VIH-1. Tot i així, per controlar la replicació del VIH en absència de TARc serà necessària la combinació amb respostes immunes potents específiques del VIH-1, com succeeix en els EEC. Futurs assajos clínics en persones amb un reservori baix de VIH-1, com els individus LoViReT o que hagin rebut un trasplantament al·logènic de cèl·lules mare, serien de gran interès en estratègies destinades a estimular les respostes immunes específiques del VIH-1 amb l'objectiu final de curar el VIH-1.

CHAPTER 1. INTRODUCTION

1.1. Human Immunodeficiency Virus

1.1.1. HIV History and Origin

In June 1981, the USA Centers for Disease Control (CDC) reported five cases of *Pneumocystis carinii* (now *P.jirovecci*) in previously healthy young men in Los Angeles[1,2]. These individuals had no previous record of disease, but all presented an impaired immune system with a depletion of CD4$^+$ T lymphocytes, unusual infections and/or cancers, such as Kaposi's sarcoma. This unknown disease was called Acquired Immune Deficiency Syndrome (AIDS). In 1983, a group of scientists headed by Françoise Barré-Sinoussi and Luc Montagnier isolated a new retrovirus from a cultured derived from a lymph node (LN) biopsy from an individual with generalized lymphadenopathy. It was named lymphadenopathy-associated virus (LAV)[3], and Barré-Sinoussi and Montagnier obtained the Nobel Prize in 2008 for this achievement. The isolation of a similar virus in individuals with AIDS confirmed the discovery of the virus causing AIDS, renamed as human T-lymphotropic virus type III (HTLV-III)[4]. Later, the virus was denominated as Human Immunodeficiency Virus (HIV) by the international Committee on the Taxonomy of Viruses[5]. In 1986, another human retrovirus related to HIV-1 was found in West African individuals and was named HIV type 2 (HIV-2) to distinguish it from the original type HIV-1[6].

Phylogenetic and statistical analyses have estimated that HIV first infected humans in the 1920s in Kinshasa[7–9] through multiple zoonotic infections, or cross-species transmissions of simian immunodeficiency virus (SIV), found in African non-human primates[10]. Specifically, HIV-1 is phylogenetically related to SIVcpz isolated from the West Central African chimpanzees (*Pan troglodytes troglodytes*)[11] whereas HIV-2 is highly associated with SIVsm from sooty mangabeys (*Cercobecu atys atys*)[12].

1.1.2 Global spread of HIV

The HIV/AIDS pandemic is a major global health issue, being one of the most devastating infectious diseases ever known. It has been responsible for nearly 76 million infections and 33 million people have died due to HIV/AIDS related complications since the start of the epidemic. In 2019, the Joint United Nations Programme on HIV/AIDS (UNAIDS) estimated that about 38 million people were

living with HIV worldwide, 1.7 million were newly infected and more than 690.000 people died from AIDS-related illnesses[13].

Despite being a worldwide pandemic, the prevalence varies greatly between continents and countries, with the biggest epidemic affecting East and Southern Africa, where 43% of all new worldwide infections take place. In this region 6.7% of the adult population is living with HIV (Fig.1).

Global HIV prevalence: 0.7%
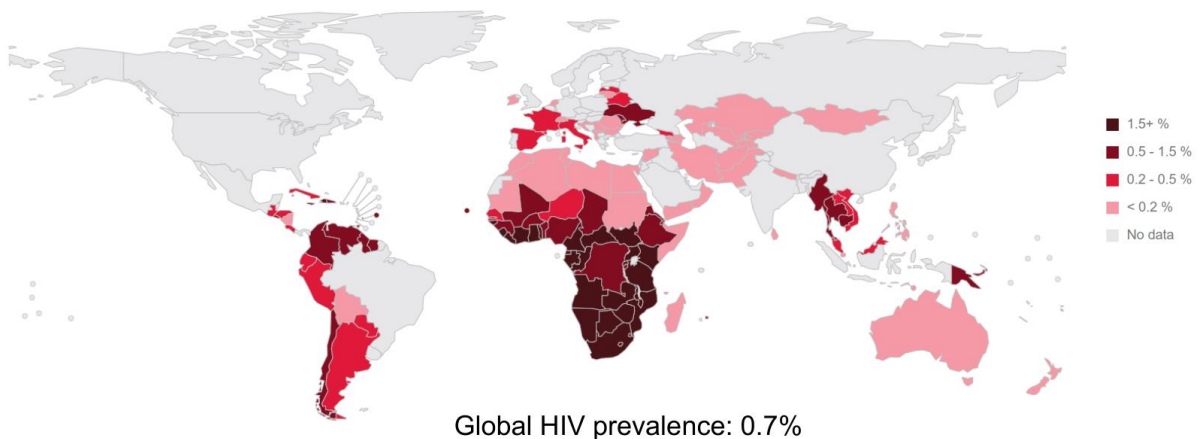
Figure 1. Adult HIV prevalence worldwide in 2019. Obtained from UNAIDS[13].

1.2. HIV Virology

1.2.1. HIV classification

HIV belongs to the group VI of reverse transcribing viruses, *Retroviridae* family, *Orthoretrovirinae* subfamily, *Lentivirus genus*[14]. Two types of HIV have been characterized, HIV-1 and HIV-2. Despite being similar, both species differ in their replicative and pathogenic capacity, virus evolution and target of infection. The most prevalent virus is HIV-1, accounting for around 95% of all infections worldwide, while HIV-2 is mainly restricted to West Africa. HIV-1 is classified into four groups: M (major or main), O (outlier) N (non-M, non-O) and P (putative). Each of these groups resulted from an independent cross-species transmission, with group M being the first to be discovered and the most common worldwide.

Phylogenetic studies have shown the existence of 10 subtypes within M group (A, B, C, D, E, F, G, H, J, and K), and several circulating recombinant forms[15,16].

Among group M, the most prevalent is the subtype C. However, in Western Europe, America, and Australia the most dominant is subtype B. As a result, the great majority of HIV clinical research has been conducted in populations where subtype B predominates.

1.2.2. HIV-1 structure and genome

HIV-1 virions are spherical particles of about 119-207nm[17] in diameter wrapped with an envelope (Fig. 2). This is formed by a lipid bilayer derived from the host cell that contains embedded trimers of the envelope glycoproteins (Env; gp160), the only virus-encoding determinants on the virus surface. Gp160 consists of a gp120 protein anchored to the membrane by the gp41 transmembrane protein[18], which are responsible for the attachment and fusion to the host cell. Attached to the inside of the envelope there is the matrix (MA), which provides the virion with structural and mechanical support. The matrix surrounds the conical-shaped capsid core (CA) and the nucleocapsid (NC). Within the capsid, there is the viral ribonucleic acid (RNA) protected by the closely associated NC. The CA also contains the protease, reverse transcriptase and integrase enzymes[19].
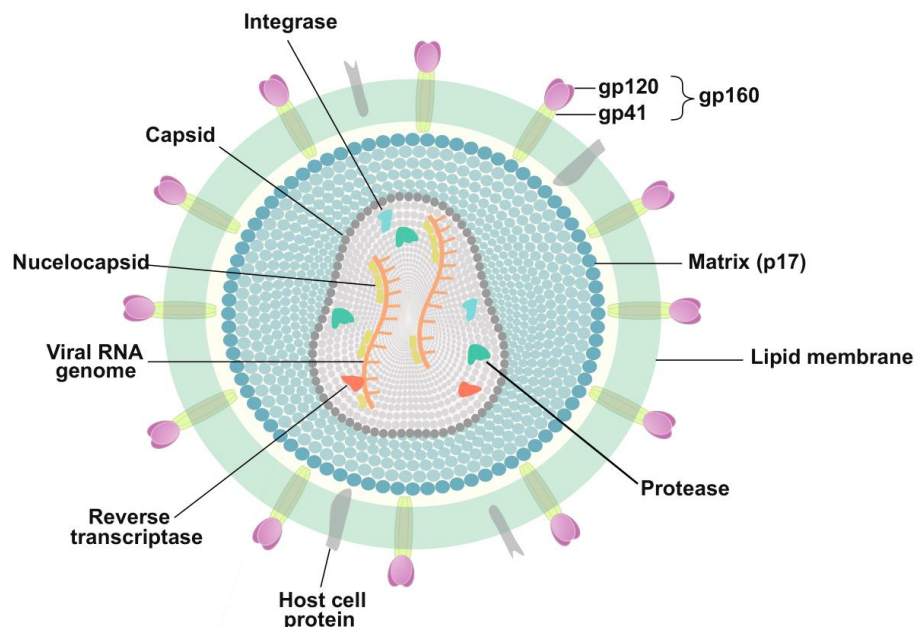
Figure 2. Schematic structure of the HIV-1 virion.

The viral genome is composed of two copies of positive single-stranded RNA (ssRNA) of approximately 9.8 kb in length[20], flanked by a repeated sequence known as the long terminal repeats (LTR). The LTR regions contain sites important for viral

integration, packaging, and transcription regulation. The HIV-1 genome encodes for nine genes: three major genes encoding for structural proteins (*gag*, *pol* and *env*), two regulatory genes (*tat* and *rev*, and four accessory genes (*vif, vpr, vpu* and *nef)* (Fig. 3).
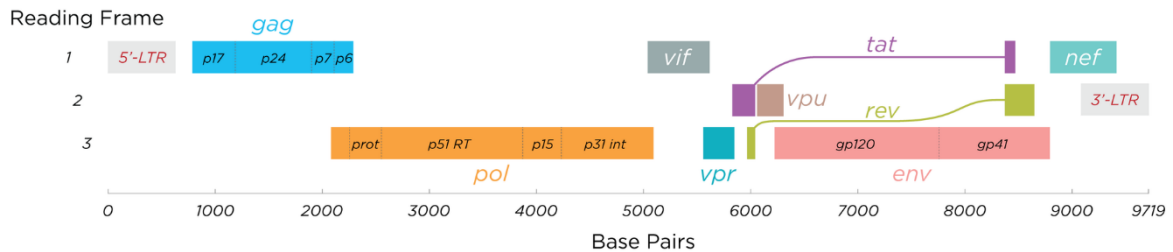
Figure 3. Schematic structure of the HIV-1 genome. Reproduced from Splettstoesser T[21].

-   Major structural genes
    -   *gag* (group specific antigen): codifies for the Gag polyprotein, which is processed during maturation to generate the structural proteins p17 or MA, p24 or CA, p7 or NC, and p6
    -   *pol* (polymerase): codifies for the viral enzymes reverse transcriptase (transcribes DNA from RNA), integrase (integrates the double-stranded RNA into the host genome) and protease (cleaves the precursor Gag polyprotein to produce structural proteins)
    -   *env*: codifies for gp160, the precursor of gp120 and gp41 proteins that are embedded into the viral envelope. Enables viral fusion with target cells
-   Regulatory genes
    -   *tat* (trans-activating regulatory protein): codifies for the protein Tat which regulates the reverse transcription of viral RNA
    -   *rev* (regulator of expression proteins): regulates the export of non-spliced and partially spliced viral mRNA from the nucleus to the cytoplasm
-   Accessory genes
    -   *vif* (virion infectivity factor): encodes the Vif protein that is critical for infectious virus production
    -   *vpr* (viral protein R): encodes the Vpr protein that facilitates the nuclear import of cDNA, transactivates HIV-1 promoters, halts cellular growth and induces cellular differentiation

o *vpu* (viral protein U): encodes the Vpu polypeptide that induces virion release from the surface of an infected cell, and is involved in CD4 degradation

o *nef* (negative factor Nef): encodes the Nef protein that has influence on HIV-1 replication, enhancement of infectivity, downregulation of CD4 on target cells

1.2.3. HIV-1 replication cycle

The HIV-1 replication cycle includes several stages and all of them can be potentially inhibited by antiretroviral drugs (Fig. 4).
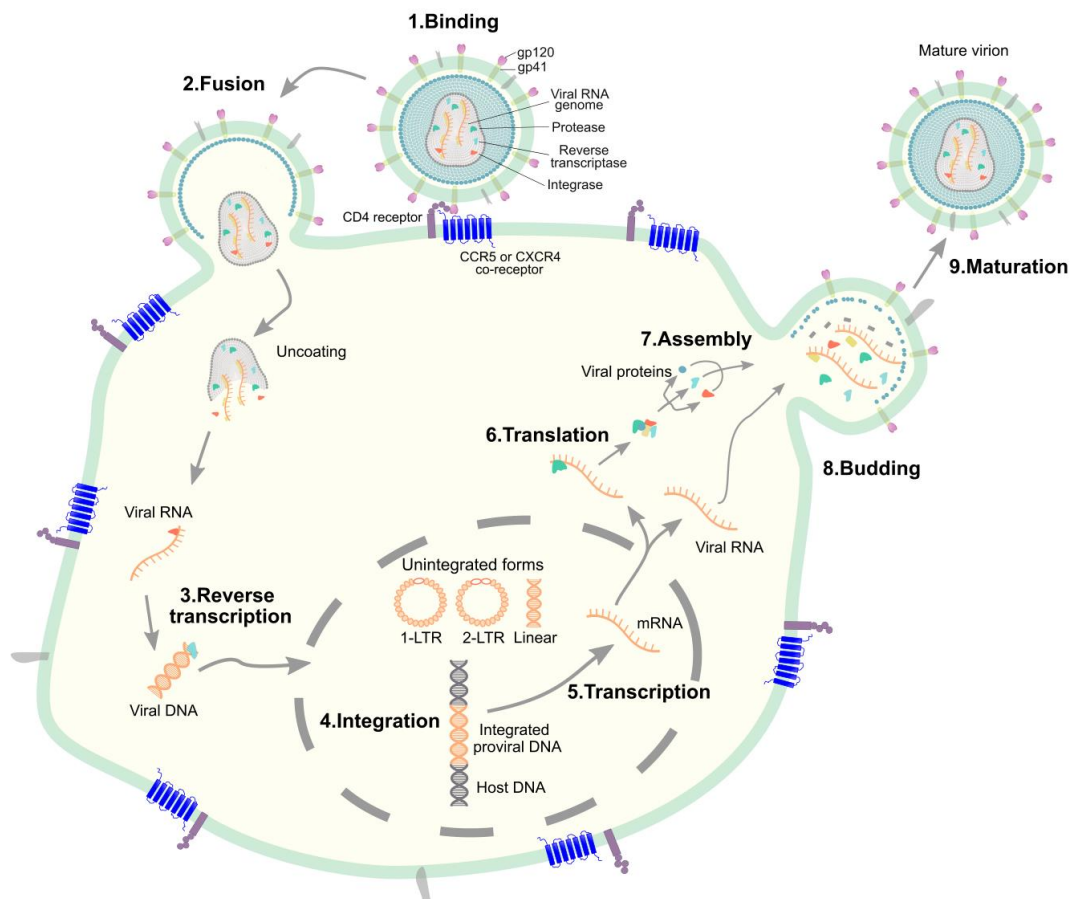
Figure 4. Schematic overview of the HIV-1 replication cycle. The figure illustrates the main steps in the HIV-1 life cycle divided in an early and a late phase. The early phase includes: (**1**) the binding of the virus particle to the CD4 receptor and co-receptors CCR5 or CXCR4; (**2**) the fusion with the host cell membrane followed by the uncoating of the viral capsid and the release of the HIV-1 RNA genome and proteins into the cytoplasm; (**3**) the reverse transcription of the viral RNA genome into a DNA duplex, the translocation into the nucleus and the (**4**) integration into the cell genome. After the integration, the late phase of the cycle starts, in which: (**5**) the proviral DNA is transcribed to form new viral RNA, which subsequently is (**6**) translated to form viral proteins; these proteins translocate to the cell surface to (**7**) assemble in the cell membrane and form new viruses. Finally, the new viral particles (**8**) bud off and suffer a process of (**9**) maturation until becoming mature virions.

Binding and fusion

The viral protein gp120 binds with the cellular receptor CD4, which is present on the surface of target cells[22]. The binding causes a conformational change in gp120 exposing the co-receptor binding site (or V3 loop) allowing gp120 to interact with the co-receptor CCR5 or CXCR4[23]. The co-receptor used for cellular entry determines the tropism of the virus, which is termed R5-, X4- or dual-tropic accordingly. Individuals lacking the CCR5 co-receptor due to a homozygous Δ32 deletion in the *CCR5* gene are resistant to infection by R5-tropic strains[24,25]. The binding of gp120 to CD4 and the co-receptor causes a conformational change in gp41, leading to the fusion between the viral and cellular membrane. Afterward, the viral capsid core is internalized, and the RNA released into the cytoplasm[26]. The entering capsid contains two copies of the viral RNA, and various enzymes, including reverse transcriptase, integrase and protease[27].

Reverse transcription

The ss-RNA is retrotranscribed by the reverse transcriptase enzyme into linear double-stranded DNA (dsDNA)[27]. Due to the absence of proofreading mechanism, this process introduces high rates of DNA base pair mutations, contributing to the high rate of viral evolution[28].

Integration

Following reverse transcription, the dsDNA is translocated into the cell nucleus and integrated into the host cell genome by the viral enzyme integrase in a two-step process[29]. The first step, which occurs in the cytoplasm, is called 3'end processing, where the integrase primes the viral DNA by removing two nucleotides from both 3'ends of the viral DNA. Then, the viral DNA binds to integrase complexes with other host cell and viral proteins to form the viral pre-integration complex (PIC). The PIC is transported into the nucleus where the viral enzyme integrase catalyzes the second step of the viral integration process known as "strand transfer", which consists of the ligation of the processed DNA into the host chromosome. Once the virus is integrated into the host genome, the HIV-1 DNA is called provirus and the host cell is permanently and irreversibly infected.

Since the integration process is not entirely efficient, aborted integration events may also be found in the nucleus. The unintegrated DNA can be found as linear or circularized DNA. Circularization is performed by host DNA repair enzymes to form episomes containing two copies of the LTR (2-LTR circles) or undergo recombination to form 1-LTR circles[30].

Transcription and translation

When the host cell receives a signal to become activated, the provirus transcribes to mRNA using the cellular polymerase II. First, HIV-1 transcripts are multi-spliced (ms) to form *tat*, *rev* and *nef* mature mRNA, which are transported into the cytoplasm to be translated into viral proteins. Then, Tat and Rev return to the nucleus where Tat increases the rate of transcription by promoting elongation. The accumulation of Rev in the nucleus leads to the transcription of single and unspliced (us) mRNAs of *gag, gag-pol, env, vif, vpr* and *vpu*. Then, all these transcripts are transported to the cytoplasm, processed and translated into viral proteins[31].

Viral assembly, budding and maturation

Assembly of new HIV-1 virions occurs at specialized plasma membrane microdomains of the host cell, mediated by the Gag and Gag-Pol polyproteins precursors, which contain the MA, CA, NC and p6 domains. The MA domain targets Gag to the plasma membrane to promote the incorporation of Env glycoproteins into the forming virions. The CA domain mediates protein-protein interactions required for immature virion assembly. The NC domain recruits the viral RNA genome into the virions and facilitate the assembly, and the p6 domain recruits the host endosomal sorting complex required for transport machinery which mediates the budding process. After the budding of the immature virion, the viral protease is activated and cleaves Gag and Gag-Pol polyproteins into their functional subunits: MA, CA, NC, and p6 proteins, converting the immature virion into its mature infectious form[32].

1.3. HIV-1 pathogenesis

1.3.1. Transmission

HIV-1 can be transmitted by the exchange of body fluids from infected people such as blood, semen, vaginal secretion, and breast milk. Also, it can be transmitted by vertical transmission from mother to child during pregnancy or delivery. The risk of infection is dependent of different factors such as the route of infection, viral concentration in the exposed body fluid, presence of other sexually transmitted diseases, and the susceptibility of the recipient. Sexual transmission through the lower genital tract and rectal mucosa represents the highest proportion of the new infections[33].

In most cases, the infection is established by a single virus, however this may vary depending on the infection route[34,35]. Specifically, during sexual transmission, cell-free or cell-associated virions can penetrate the epithelium through microabrasions or by transcytosis. Then, the virus rapidly reaches dendritic cells (DCs), Langerhans cells, intraepithelial CD4$^+$ T cells and resting CD4$^+$ T cells in the lamina propria[36,37] . After local viral expansion, the virus disseminates to the draining LN, and subsequently arrives to the bloodstream to establish the infection into secondary lymphoid organs, with particularly preference for the CD4$^+$ T cells located in the gut associated lymphoid tissue (GALT)[38].

1.3.2. Natural course of HIV-1 infection

HIV-1 infection is characterized by a progressive decline in the number of CD4$^+$ T cells, rise in the HIV-1 viral load, chronic immune activation, and exhaustion of the immune system. In the absence of treatment, an asymptomatic period of 8 to 10 years is observed before the progression to AIDS[39], which is associated with the development of opportunistic infections and cancers that finally leads to death[40]. The natural course of infection commonly includes three different stages (Fig. 5): acute phase, chronic phase, and AIDS.
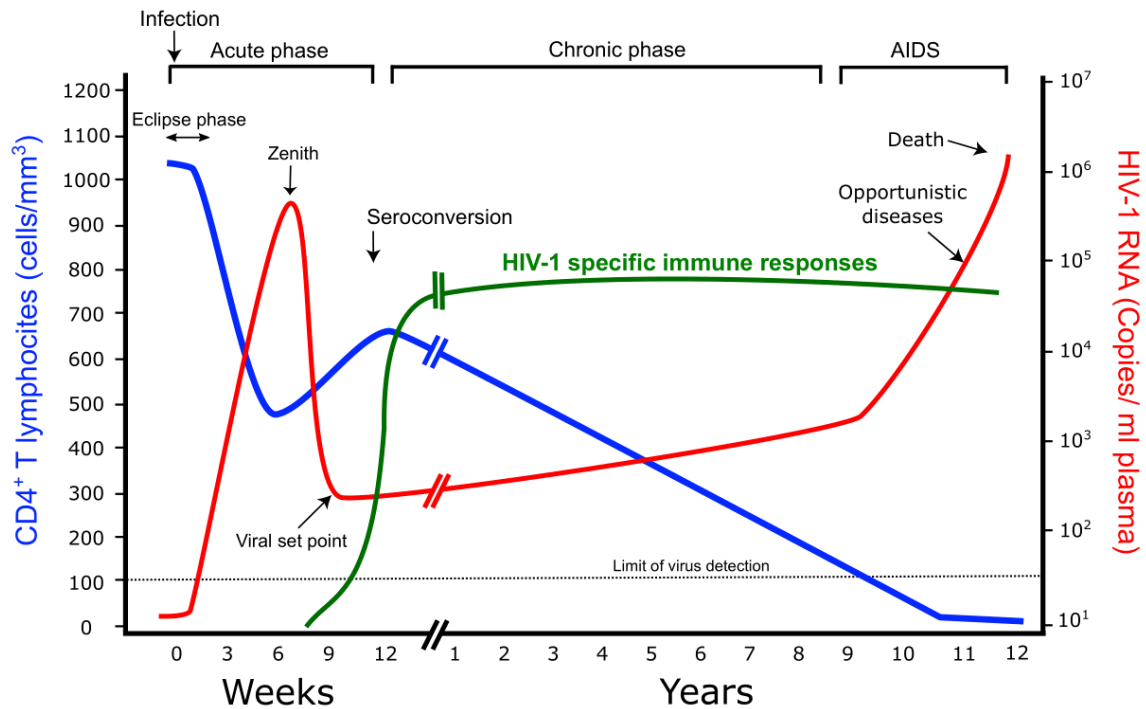
Figure 5. Schematic representation of the natural HIV-1 course of infection in the absence of antiretroviral therapy. CD4+ T lymphocytes count is shown in blue, HIV-1 RNA copies/ml in red, and HIV-1 specific immune responses in green. Adapted from Splettstoesser T[21].

Acute infection

This phase lasts from 2 to 9 weeks after infection. The end is marked by the detection of HIV-1 antibodies in plasma, which is called seroconversion.

The first days after HIV-1 infection the virus replicates and spreads to different tissues and organs, but viremia is still undetectable in plasma and there is no immune response or symptoms, generating what is known as the eclipse phase[41]. After this period, still in the acute phase, there is an increase in plasma viremia, reaching the zenith around day 21-28 (10^5-10^7 HIV-1 RNA copies/ml plasma), with a consequent massive depletion of CD4+ T cells. The depletion in immune cells is the result of the viral cytopathic effect and host immune response. During this phase, HIV-1-infected individuals may develop a virus-like illness (acute retroviral syndrome) characterized by fever, pharyngitis, lymphadenopathy, and rash.

After the peak of plasma viremia, the immune system generates antibodies (seroconversion) against HIV-1 viral proteins and CD8+ T cell responses against HIV-1 antigens expressed on the surface of infected cells. At the end of acute

infection, the immune responses will reduce plasma viremia reaching a virological set point (predictor of disease progression) and increase the levels of CD4$^+$ T cells.

Chronic infection

Acute HIV-1 infection is followed by a chronic and generally asymptomatic phase of the infection that lasts from 8 to 10 years[39], and is characterized by a continuous and slow decline of peripheral CD4$^+$ T cells and stable viral loads. In this phase there is a constant stimulation of the immune system due to the persistent exposure to viral antigens and altered cytokine environment. However, the virus continues replicating and disseminates into lymphoid organs.

AIDS

This phase is characterized by a drop of CD4$^+$ T cell counts bellow 200 cells/mm^3, a dramatic increase of viral load, failure in the immune responses, and lymphoid architecture is destroyed. Therefore, this enables the onset of AIDS-defining illnesses such as opportunistic infections, HIV-1 associated cancers, and kidney failure, which eventually results in death.

1.3.2.1. Alternative HIV-1 progression

Although the 80-90% of HIV-1-infected individuals present a natural history of the infection as it was just described, a fraction is able to remain clinically stable and without progression to AIDS in the absence of antiretroviral treatment. These individuals represent between 5 to 15% of the total HIV-1-infected population and are termed as long-term non progressors (**LTNPs**), which remain clinically and/or immunologically stable for more than 10 years with CD4$^+$ T cell counts over 500 cells/mm^3 [42–45] (Fig. 6). Among LTNP, we found elite controllers (**EC**) which have the ability to maintain plasma viremia to undetectable levels (<50 copies/ml) and generally maintain elevated CD4$^+$ T cell counts (200 to 1000 cells/mm^3) for at least 1 year , and represent less than 1% of the total HIV-1-infected population[46–48]. An additional progression phenotype is the called viremic controllers (**VC**), whom achieve a less degree of virologic control (<2000 copies/ml) while maintaining elevated CD4$^+$ T cell counts (≤500 cells/mm^3)[49,50].
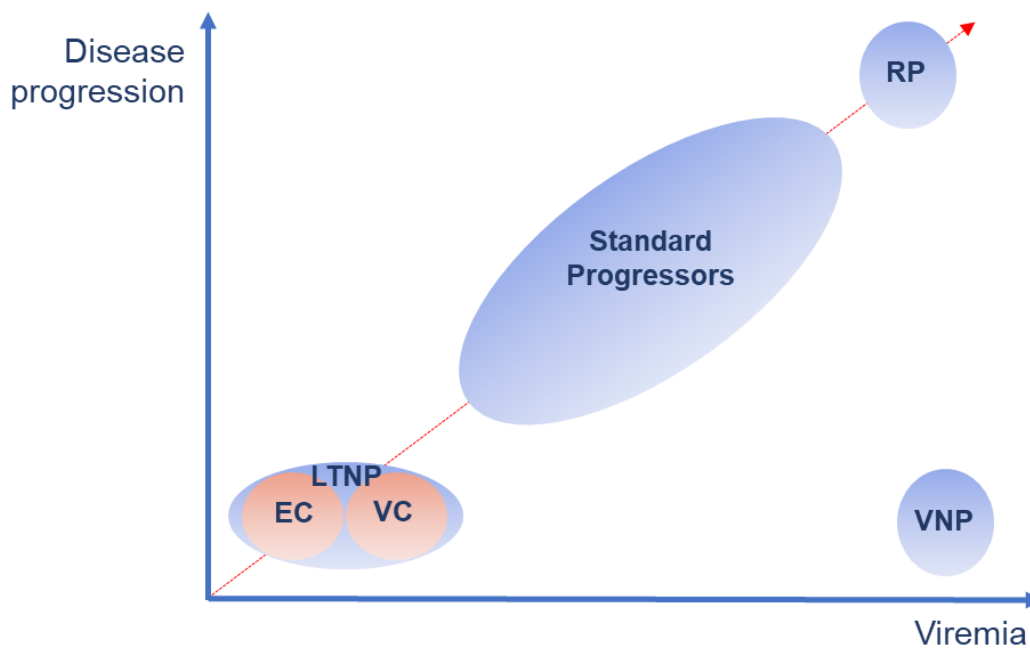
Figure 6. Schematic representation of the variation in HIV-1 disease progression. EC: Elite Controllers; LTNP: Long Term Non progressors; VC: viremic controllers; VNP: Viremic non progressors; RP: Rapid progressors. Adapted from Telenti A and Johnson WE[51]

On the other extreme of disease spectrum, exists a small group of HIV-1-infected individuals (<0.5%) with a rapid progression to AIDS within 2-3 years after primary infection, the rapid progressors (**RP**) (Fig. 6). They may present clinically severe acute infection, and are defined by a decay of CD4$^+$ T cells below 350 cells/mm^3 within 3 years after seroconversion, with, in most cases, high levels of plasma viremia[42,52].

Finally, there are individuals termed as viremic non-progressors (**VNP**) characterized by having high levels of HIV-1 replication during the chronic phase of infection despite remaining asymptomatic, and maintain high CD4$^+$ T cell counts for long periods of time in the absence of antiretroviral treatment[53] (Fig. 6).

1.4. Antiretroviral treatment of HIV-1 infection

The development of antiretroviral treatment has modified the natural course of the HIV-1 infection achieving one of the most significant advances in the HIV-1 field. AZidoThymidine (AZT) was the first antiretroviral approved in 1987. It was the first of a family of nucleoside reverse transcriptase inhibitor (NRTI) that interferes with HIV-1 replication by blocking the reverse transcriptase[54]. During several years, NRTI
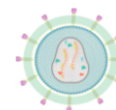
and a parallel group of non-nucleoside reverse transcriptase inhibitors (NNRTI) were administered as mono or dual therapy, leading to the development of drug resistances due to a failure of a completely suppression of viral replication. In 1996, long-term suppression of HIV-1 was achieved by the administration of triple-drug therapy, called highly active antiretroviral therapy (HAART) or combined antiretroviral therapy (cART)[55,56].

To date, more than 40 drugs have been approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of HIV-1[57]. These drugs are classified by the mode of action in six categories:

1. Nucleoside/nucleotide-analog reverse transcriptase inhibitors (NRTIs): inhibition of the viral DNA transcription by incorporation of a defective nucleotide.
2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs): inhibition of reverse transcriptase by binding to its active site
3. Integrase inhibitors (INIs): prevents integration of viral DNA into the host genome
4. Protease inhibitors (PIs): inhibition of protease enzyme resulting in non-mature virions
5. Fusion inhibitors: interfere with binding, fusion, and entry of HIV-1 to the host cell
6. Entry inhibitors: blocks binding of HIV-1 to the co-receptor CCR5 or CD4 receptor

Typically, a standard cART consists of a combination of 2 NRTI and one drug from any of the other categories. cART suppresses viremia below the clinical detection limit, restores CD4$^+$ T cell counts, reduces the risk of comorbidities, and increases the life expectancy of HIV-1-infected individuals near the general population[58]. Also, it has been shown that if a highly adherence to daily therapy exists, the risk of viral transmission is reduced to negligible levels[59].

Nowadays it is recommended to start cART immediately upon diagnosis of HIV-1, regardless of CD4$^+$ T counts or viral load, since it has been associated with an improved prognosis[55]. Furthermore, several studies indicate that starting cART

during acute or early HIV-1 infection compared to during chronic infection have additional benefits such as lower progression, preserved CD4$^+$ T cell counts, reduced viral set point, and less destruction of the lymphoid tissue[60].

1.5. HIV-1 persistence during suppressive antiretroviral therapy

cART is effective and reduces HIV-1 RNA levels below the limit of detection (LOD), reconstitutes the immune system, decreases the morbidity, mortality, and viral transmission[58,59] but it is not able to cure the infection. If treatment is interrupted, in most cases, HIV-1 viral load rebounds in a median of 2 to 8 weeks[61] with a decrease in the CD4$^+$ T cell count. In 1995, it was demonstrated for the first time that the reason why HIV-1 cannot be cured was that resting CD4$^+$ T cells from HIV-1-infected individuals contained integrated HIV-1 DNA[62]. That phenomenon was known as latent infection.

Latent infection is a reversibly non-productive state of HIV-1 infection, which is characterized by the presence of infected cells that are transcriptionally silent but capable of being induced to produce replication-competent virus. Alternatively, a reservoir is defined as a cell type or anatomical site in which virus accumulates and persist[63].

The reservoir levels fluctuate and evolve during the natural and therapeutic course of HIV-1 infection. Thus, reservoirs can be detected very early after infection with median total blood HIV-1 DNA load of 3.3 log$_{10}$ copies/10^6 PBMC after a median of 47 days after infection[64]. Once cART is started, the reservoir in blood is reduced, being variable among different individuals[65–68]. Specifically, individuals who naturally control HIV-1 infection present a very low and stable total HIV-1 DNA levels in the absence of cART[48,69–71]. Reduced latency is also observed in the called post-treatment controllers (PTC), in whom treatment was initiated early during the primary infection, but long-term viral control after treatment interruption was maintained[72]. On the other hand, individuals who do not control HIV-1 replication or are symptomatic have significantly higher HIV-1 DNA levels[73]. However, the mechanism by how the reservoir is stablished and how varies among individuals is still unknown.

1.5.1. Cellular and anatomical HIV-1 reservoirs

Since the first description of resting CD4^{+} T cells as a reservoir for HIV-1[62], different cell types and tissue compartments have been described as reservoirs. Consequently, HIV-1 reservoirs are diverse and can be conceptualized as either cellular or anatomical reservoirs (Fig. 7).
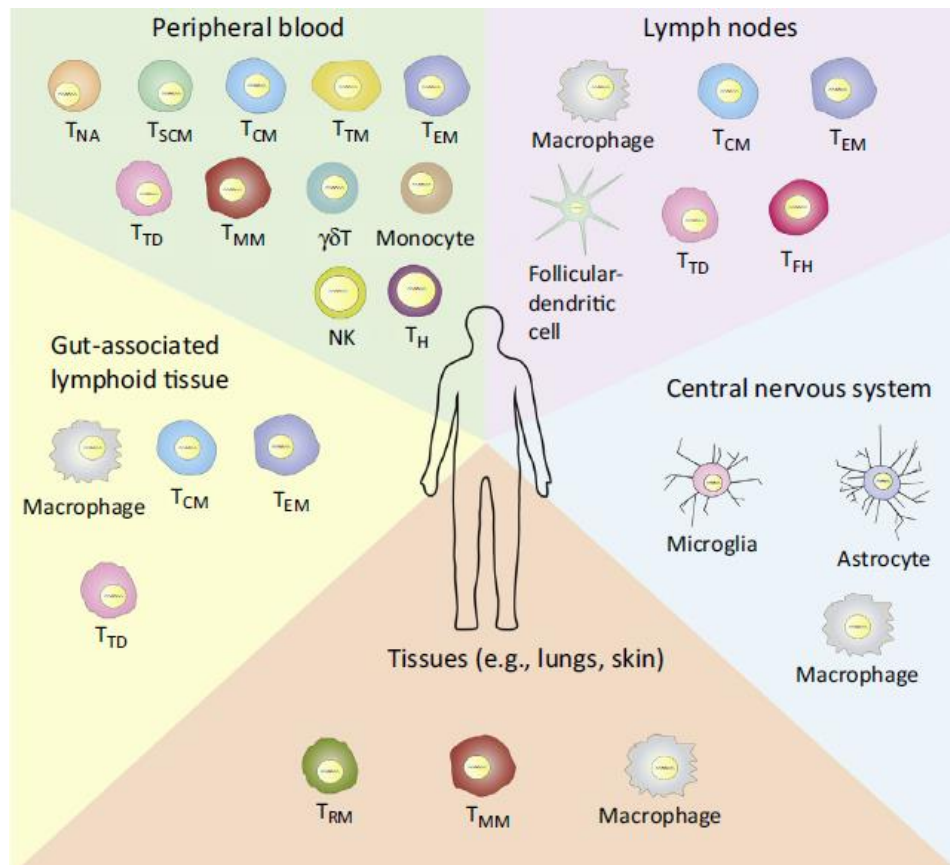
Figure 7. Schematic representation of tissues and specific tissue-cells contributing to the persistence of HIV-1 during effective cART. Reproduced from Barton K. *et al*[74].

1.5.1.1. Cellular reservoirs

HIV-1 primarily infects CD4^{+} T cells due to the need of specific interaction of gp120 with the CD4 receptor to the virus entry. In particular, memory CD4^{+} T cells are the largest contributor to the HIV-1 reservoir and the most widely characterized population[75]. There are different types of memory CD4^{+} T cells, based on their memory and functional status (Fig. 8). Several studies have shown that CD4^{+} T cell subsets harbor different amounts of latent HIV-1 and the mechanisms by which support viral persistence might be different. T central memory (T_{CM}), T transitional memory (T_{TM}), and T effector memory (T_{EM}) CD4^{+} T cells contain HIV-1 DNA at

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higher frequencies than the more differentiated subsets, such as T terminally differentiated (T_{TD}) cells[75]. A subset with stem cell like properties, the T stem cell memory (T_{SCM}) cells have been shown to significantly contribute to the HIV-1 reservoir due to their increase proliferative capacity and self-renewal, although their levels of total HIV-1 DNA and their proportion in peripheral blood are lower than other memory CD4+ T cell subsets[76]. Naïve CD4+ T cells (T_N) also contain HIV-1 DNA, but at lower frequency than the other mentioned T cell subpopulations[75]
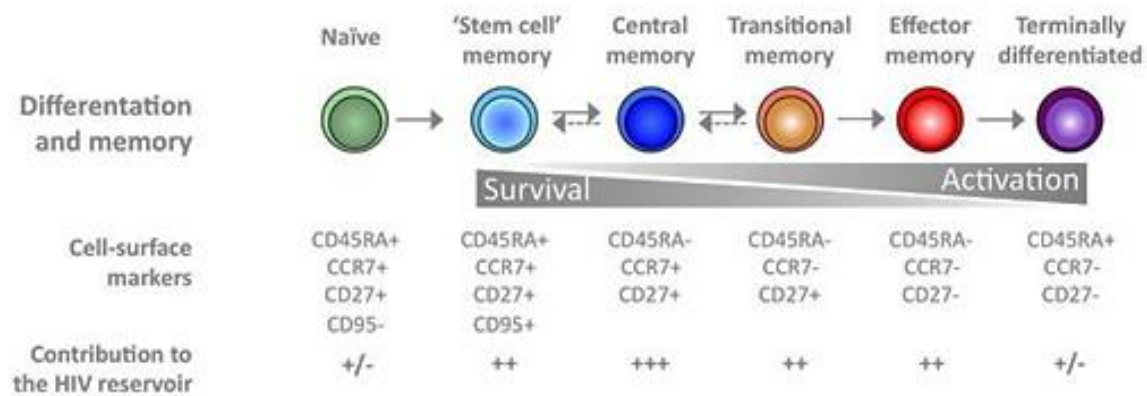
Figure 8. Contribution of CD4+ T cell subsets to the HIV-1 reservoir during cART. CD4+ T cell subsets can be classified according to their differentiation and memory status[77].

Other CD4+ T cell subtypes, such as T follicular helper (T_{FH})[78–80], T regulatory (T_{reg})[81,82] , tissue resident memory (T_{RM})[83,84] and T migratory memory (T_{TM}) (Fig. 7) cells also contribute to the long-lived HIV-1 reservoir.

Additionally, other cell types in the myeloid lineage such as monocytes, macrophages and DCs have been proposed to be permissive for HIV-1 infection. The expression of the CD4 receptor on these cells is much lower than on CD4+ T lymphocytes, and their ability to get infected remains controversial. Monocytes are derived from myeloid progenitors in the bone marrow and they are the precursors of macrophages and DCs. HIV-1 has been rarely detected in circulating monocytes of HIV-1-infected individuals[85,86]. Moreover, given their short half-life of a few days before their differentiation into macrophages and their high turnover suggest that they do not represent a stable reservoir, but could represent an important viral reservoir for their ability to disseminate into different tissues and differentiate in macrophages. In fact, in macrophages from different organs (microglial cells, alveolar macrophages, intestinal macrophages…) have also been detected HIV-1

DNA[87–89]. However, it was not until 2019 the demonstration that macrophages harvested from urethral tissue of individuals under cART harbor replication-competent HIV-1[90]. On the other hand, in DCs low levels of proviral DNA has been observed *in vivo*, however the most important contribution of DCs to the HIV-1 reservoir seems to be the ability to retain HIV-1 virions and transfer it to CD4+ T cells[91,92].

1.5.1.1. Anatomical reservoirs

HIV-1 reservoirs have been widely analyzed in peripheral blood, but only 2% of the total amount of lymphocytes are found in there[93]. Therefore, a different scenario could be happening in tissues, making important the study of the HIV-1 reservoir in different anatomic compartments.

The cells previously described that contain HIV-1 DNA can be found in different tissues or anatomic compartments such as lymphoid tissues, brain, kidneys, lung, liver, adipose tissue, gastrointestinal tract, genitourinary system, and bone marrow (BM) (Fig. 7)[94,95]. The lymphoid tissues, which comprises the spleen, thymus, LN and GALT, are the most important sites of viral replication during active infection and HIV-1 DNA is still detected after years of cART[95]. In many cases, these anatomical reservoirs have a less efficient drug penetration and/or poorer immune surveillance by the effector cells of the immune system[96]. Thus, viral replication and viral reseeding may occur.

GALT is the largest lymphoid tissue in the body and comprises the largest population of T cells and macrophages[93]. It is thought to be the main primary site of initial HIV-1 replication and an important contributor to the overall pool of latently infected CD4+ T cells during treated HIV-1 infection. Several studies have shown that it contain much higher levels of HIV-1 DNA than in peripheral blood among HIV-1-infected individuals under cART[97,98]. One study by Yukl *et al* estimated that the gut contains 1.2×10^9 infected CD4+ T cells, which would represent the 83-95% of all HIV-1-infected cells in the body[99].

Other secondary lymphoid tissues such as LNs or spleen represent an important HIV-1 reservoir. They are the primary site for viral replication and contain high numbers of infected cells and free virions[100]. Although cART can decrease in 3 logs

the HIV-1 RNA in LNs, HIV-1 DNA and -RNA can still be detected in individuals after long periods of cART[101–103]. In fact, several studies have shown a higher concentration of HIV-1 DNA and -RNA in LNs and spleen compared to peripheral blood[104]. In LNs, rather than being the T_{CM} and T_{TM} subsets the largest proportion of the infected cells as in peripheral blood, the memory subset T_{FH} (characterized by the expression of C-X-C chemokine receptor type 5 (CXCR5) and Programmed cell death protein 1 (PD-1)) is the most enriched subpopulation for replication competent virus and viral RNA[78].

1.5.2. Reservoir establishment and maintenance

The HIV-1 reservoir is established in the first days of infection[68]. Therefore, the treatment during acute HIV-1 infection does not prevent from the establishment of a pool of latently infected CD4^{+} T cells, even in the Fiebig I stage[68].

The in vivo mechanisms responsible of the establishment of the HIV-1 reservoir are not completely clear yet. Two main models of latency have been described by which HIV-1 establishes a pool of latently HIV-1 infected cells: through the infection of activated CD4^{+} T cells that survive and revert to a memory phenotype, or via direct infection and integration into the genome of resting memory CD4^{+} T cells. *In vitro* models have shown that activated CD4^{+} T cells that are transitioning to a resting state are more permissive to HIV-1 infection, hence escaping the rapid destruction of the infected T cells[105]. Therefore, latent HIV-1 infection can be established in resting and activated cells, but the probability of establishing latency could be higher in resting CD4^{+} T cells while productive infection is more likely in activated CD4^{+} T cells[106].

Once the reservoir is established by either of these ways, the pool of latently infected cells is extremely stable, with a medium half-life of 3.7 years in HIV-1-infected individuals on cART[107,108], estimating that it would take more than 60 years of cART to eradicate the infection. Hence, the maintenance of this remarkable stable reservoir might be the result of normal homeostatic mechanisms among other factors, as showed in Fig. 9. The first explanation to the maintenance of the reservoir is the fact that the latent reservoir is composed by <u>long survival cells,</u> which are able to maintain the size of the latent reservoir for long periods of time. Also, it can be

maintained by proliferation of latently infected cells by <u>clonal expansion</u> of cells with an HIV-1 provirus integrated in genes associated with cell cycle passing identical proviral copies from dividing cells to daughter cells[109–111], by <u>homeostatic proliferation</u> (driven by cytokines such as IL-7[75] or IL-15[112]) or by some degree of <u>antigen-driven proliferation</u>, which may induce proliferation of T cells without activation of viral gene expression. Finally, it can be maintained by the low levels of residual <u>viral replication</u> that are present in some HIV-1-infected individuals[113] despite being under cART. This low level replication might be due to the insufficient drug penetration in some tissues, such as LNs[114], or cell-to-cell spread.
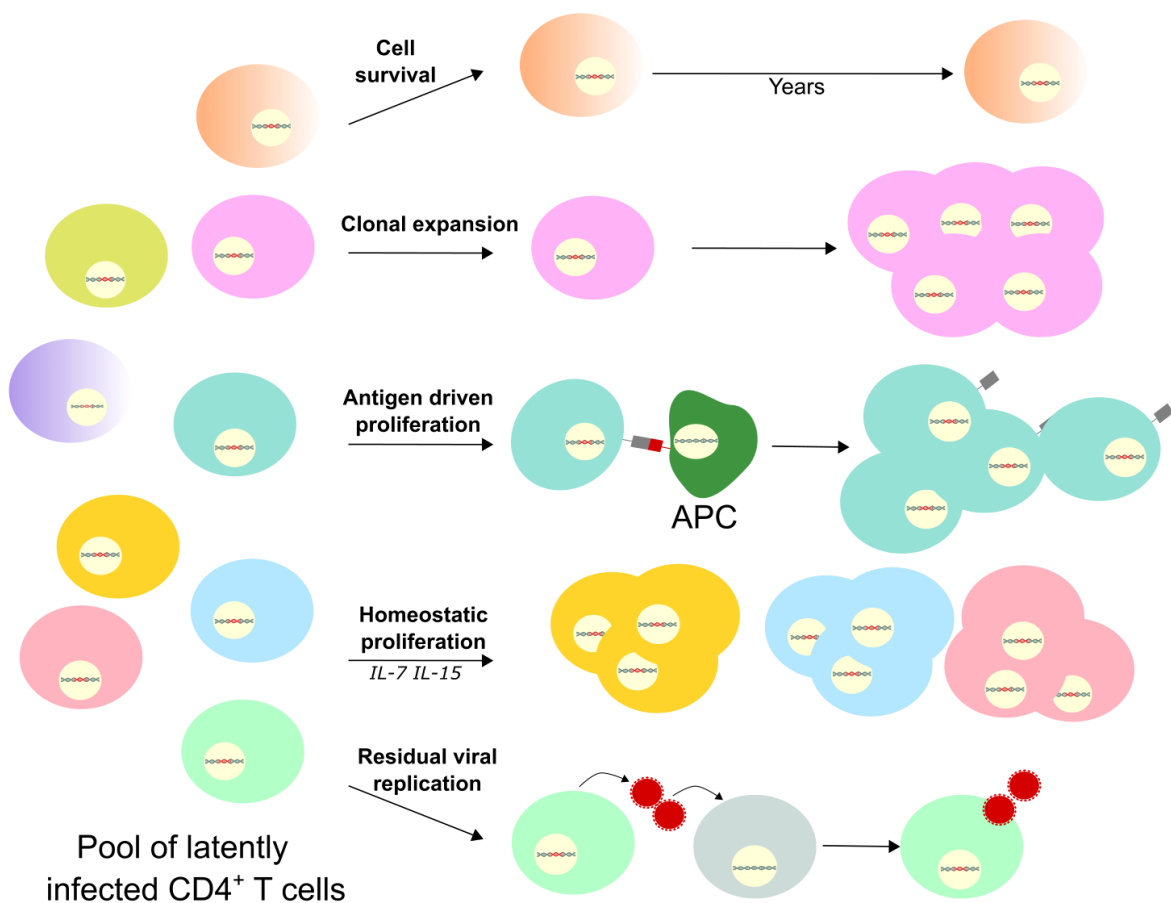
Figure 9. Different mechanisms by which the HIV-1 reservoir is maintained. Each cell color represents a different HIV-1 clone, where HIV-1 is integrated in a different part of the human genome.

1.5.3. Measuring the HIV-1 reservoir

In HIV-1-infected individuals, approximately 1 in 10^4-10^7 CD4$^+$ T cells are latently infected[115], with a rate of infection depending on the moment when cART was initiated after infection and the HIV-1 viral load set point[116]. Given these low

frequencies, the assays to measure the HIV-1 reservoirs need to be sensitive enough to detect small number of cells in a background of a large number of cells, specific to detect that a rare event is true, able to distinguish between replication-competent and no replication-competent proviruses, and precise to detect a reduction in the HIV-1 reservoir when assessing an eradication strategy.

To date, there has been developed several assays for measuring HIV-1 persistence. The different assays can measure free virus, cell-associated HIV-1 DNA (integrated or unintegrated, total or intact), the transcription-competent reservoir, the translation-competent reservoir or the replication-competent reservoir. These assays are basically based on polymerase chain reaction (PCR), flow cytometry, microscopy, and cell culture (Fig. 10). The use of each assay depends on the aim of the study, the type and quantity of sample available with the consequent advantages and disadvantages in each one (Table 1).
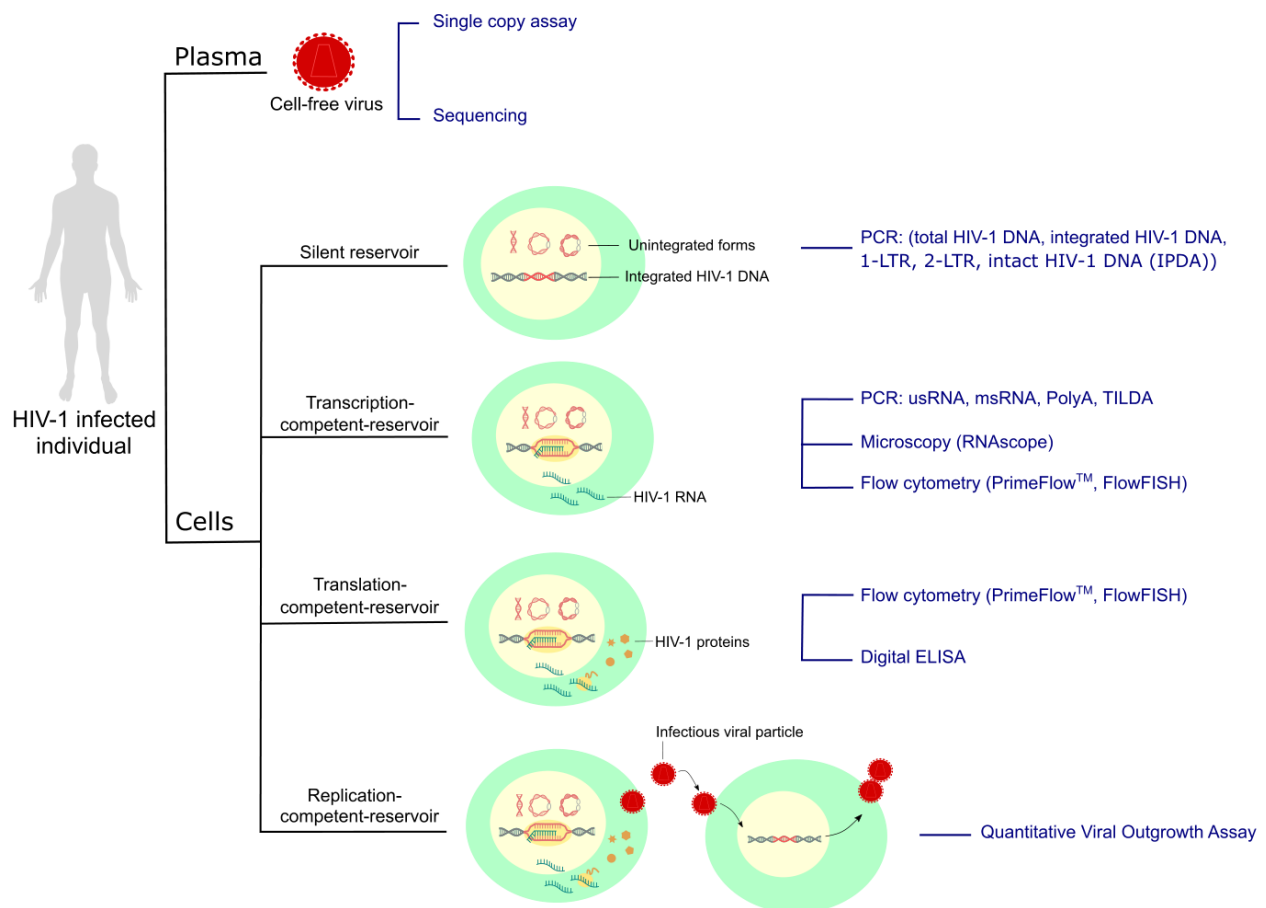
Figure 10. Assays to measure the HIV-1 reservoir.
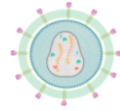
Table 1. Characteristics of assays to measure persistent HIV-1

Measurement	Assay	Detection method	Cell number requirement	Specific for intact provirus	Over/under estimation	Pros	Cons
Free virus	SCA	qRT-PCR	-	No	-	Measure residual virus production during cART	Time consuming and does not measure the latent reservoir. Large volume of body fluid
	Sequencing	PCR	-	Yes	-	Indication of the source viremia	Time consuming and does not measure the latent reservoir
Cell-associated HIV-1 DNA	Total HIV-1 DNA	qPCR or ddPCR	Low	No	Over	Easy, fast and cheap	Overestimates the size of the reservoir because it detects intact and defective provirus
	IPDA	ddPCR	Low	Yes	Over	Easy and gives an estimation of intact provirus	Primer mismatches in some individuals.
	Integrated HIV-1 DNA	qPCR	Low	No	Over	Easy and sensitive for measure of HIV persistence	Overestimates the size of the reservoir because it detects intact and defective provirus
	Non-integrated forms	qPCR or ddPCR	Low	No	Over	Marker of residual viral replication	Measurement of 2-LTR at a single point does not provide information on recency of cellular infection
Transcription-competent reservoir	caHIV-1 RNA	qRT-PCR or ddPCR	Low	No	Over	Easy and cheap	May miss cells that harbor transcriptionally silent proviruses
	TILDA	qRT-PCR	Medium	No	Over	No need for outgrowth, faster than qVOA	May detect some defective provirus
	RNA scope	Microscope	Medium	No	Over	Tissue level information, highly sensitive and specific	Limited cell phenotyping
	Flow cytometry	Flow cytometry	Medium	No	Over	High throughput, in depth phenotyping of single cells	Background in HIV-uninfected individuals, labor intensive and high starting cell number
Translation-competent reservoir	Flow cytometry	Flow cytometry	Medium	Partial	Over	High throughput, in depth phenotyping of single cells	Labor intensive, high starting cell number
	Digital ELISA	SIMOA	Medium	Partial	Over	Highly sensitive	Can detect defective provirus
Replication-competent reservoir	qVOA	p24 antigen ELISA in supernatant	High	Yes	Under	Measures replication-competent provirus	Labor intensive, large number of cells and not all intact provirus get reactivated
	mVOA	qRT-PCR	High	Yes	Under	Large number of cells can be assayed	Labor intensive and high cost. Variable levels of cell engraftment. Not all intact provirus gets reactivated

1.5.3.1. Cell-free virus

Although cell-free viruses are not considered as part of the latent HIV-1 reservoir, are a good marker of residual virus production. HIV-1 RNA from cell-free virus can be detected in body fluids (cerebrospinal fluids (CSF) and plasma) in individuals under cART, reflecting that residual viral replication exists[117].

Clinical assays can detect viremia down to 20 copies/ml, which is useful for clinical follow-up, but do not have the sensitivity to measure low-level persistent viremia (<20 copies/ml). For that reason, it was developed an assay that can detect down to one copy/ml called single-copy assay (SCA)[118]. This assay consists of the ultracentrifugation of up to 9ml of plasma or CSF, followed by manually RNA extraction and reverse transcription quantitative PCR (RT-qPCR) assay with a standard control. Modifications of this assay have been done to be less time consuming and more automated. The main differences with SCA are an automated RNA extraction, followed by RT-qPCR using the Abbott Real-Time HIV-1 assay (Abbott assay) or the RocheCOBAS® AmpliPrep/COBAS® TaqMan® HIV-1 test, v. 2.0 (TaqMan® test v2.0) [119–123] marked by an adjusted standard curve[119].

All these assays have in common the large volume of body fluid required, the use of primers located in conserved regions of the viral genome, and a LOD of 1 copy of HIV-1 RNA/ml (or lower depending of the input volume).

Alternatively, the free virus can be extracted and sequenced, and provide an indication of the source of the viremia[124]. Moreover, if the sequence is on single-genome it can provide genetic composition and diversity of the virions present in the individual[124–126].

1.5.3.2. Cell-associated HIV-1 DNA

In the HIV-1 replication cycle, there are different forms of cell-associated HIV-1 DNA that can be targeted individually or totally to estimate the size of the latent reservoir in HIV-1-infected individuals. These include:

– Integrated: HIV-1 is integrated into the host genome, preferentially into transcriptionally active open chromatin. There is a strong preference for integration of HIV-1 into, or close to *Alu* elements[127]. The *Alu* elements are the

most numerous repetitive elements in the human DNA and are randomly
distributed

- Unintegrated:
    o Linear: the most abundant form of non-integrated DNA. It is the direct
      product of reverse transcribed viral RNA
    o Circular:
        ▪ 1-long terminal repeat (1-LTR): formed through homologous
          recombination of linear DNA at the LTR, resulting in a circular
          DNA bearing one copy of the viral LTR
        ▪ 2-long terminal repeat (2-LTR): product of non-homologous end
          joining DNA repair events that are mediated in the nucleus as a
          protective host response to the presence of ds-DNA

PCR-based assays are the most sensitive, quick, easy, and cost-effective to
measure the levels of each form of cell-associated HIV-1 DNA.

The most widely used method to quantify **total HIV-1 DNA** is by quantitative PCR
(qPCR), which includes both integrated and unintegrated forms. qPCR is performed
on DNA extracts from peripheral blood mononuclear cells (PBMCs) or CD4$^+$ T
lymphocytes, using primers/probe sets that amplify conserved regions of the HIV-1
genome, usually from *gag,* LTR or *pol* regions[128,129]. However, traditionally qPCR
requires a standard curve to estimate the total HIV-1 DNA copies to normalize the
total cell number by using a cellular gene. Moreover, its accuracy at low copy
numbers is limited by the exponential amplification of noise. For that reason, droplet
digital PCR (ddPCR) is used as an alternative to qPCR, which have greater
precision, an improved accuracy, direct quantification, and greater tolerance for
primer/probe mismatches[130,131]. ddPCR is based on the fractioning of the sample,
in this case DNA, into 20.000 droplets where the PCR amplification occurs in each
individual droplet and is then analyzed (Fig. 11). The only weakness of ddPCR is
the presence of false positive signal in levels close to the limit of detection. However,
the quantification of total HIV-1 DNA, by both qPCR and ddPCR, overestimates the

frequency of replication-competent reservoir[128] and do not provide information about
their inducibility or intactness (Table 1).

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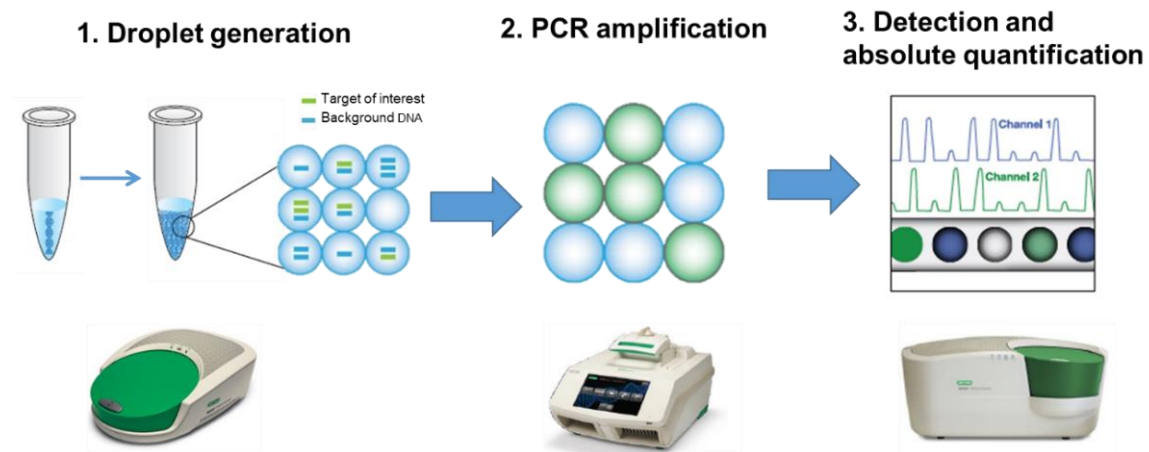
Figure 11. Schematic representation of droplet digital polymerase chain reaction (ddPCR).

Total HIV-1 DNA is based in the amplification of only one highly conserved proviral region to detect HIV-1 genomes, but it has been proved that 88.3% of the proviruses are defective or mutated[132]. Therefore, it has been recently developed an assay to detect intact proviruses, called **Intact Proviral DNA Assay** (IPDA). This technique is developed in the fact that there are different conserved regions of the HIV-1 genome that need to mute at the same time, or are always missing in a defective virus[133]. IPDA is based on a ddPCR multiplex technology, which measures the presence of an intact packaging signal (PS) and the Rev-responsive element (RRE) within *env*. It is estimated that of the proviruses amplified by IPDA, 60-70% are intact[133]. However, with the current primers described for this assay, for some individuals no amplification occurs for PS and env probes, which is attributed to sequence polymorphisms in proviral genomes. Additionally, it has been developed an assay called Q4PCR that also has the aim to distinguish intact provirus. In this case, Q4PCR is based on a quantitative PCR covering four regions of the genome followed by sequence verification of positive reactions, which provides information on proviral integrity by viral sequences[134].

Alternatively, specific assays have been developed to quantify **integrated HIV-1 DNA**. The assay most used for measuring it is the *Alu* PCR that consists of a primer targeting *Alu* elements in the human genome while the other primer targets *gag* or LTR, followed by a nested PCR for the HIV-1 LTR region to enhance sensitivity[135,136]. However, this assay has the drawbacks that requires a standard curve, has variable efficiency depending on the proximity of human *Alu* sequence

and the HIV-1 genome, and is labor intensive (high number of replicates for accurate frequency estimation) (Table 1). Other methods to quantify integrated HIV-1 DNA includes gel-fractionation[137], inverse PCR[138] and linker ligation PCR[139].

On the other hand, **unintegrated forms of DNA** (1-LTR and 2-LTR circles) can be detected in infected cells as a result of a failed integration. Although they are not considered a part of the latent reservoir, they have been used as a surrogate marker of recent infection events during cART due to their short-lived nature[65,140]. It can be measured by using primers flanking the 2-LTR circle junction, by qPCR and ddPCR[128,140–142].

1.5.3.3. Transcription-competent reservoir

Cell-associated HIV-1 RNA (**caHIV-1 RNA**) is an indicative of which cells are actively transcribing virus, and therefore can be used as a biomarker of latency reversal, but not as an indicative of the size of the reservoir.

Proviruses that are transcriptionally-competent are more likely to be replication-competent, but there are also defective viruses than can express RNA[143]. The RNA measured can be either un-spliced (us) or multi-spliced (ms), where us-RNA corresponds to the first HIV-1 mRNA transcribed and msRNA the HIV-1 mRNA processed for being translated.

Different assays exist to measure the different forms of caHIV-1 RNA, with all of them having in common the detection of the viral RNA but by different methods including amplification by PCR, RNA staining and detection by flow cytometry or microscopy. Moreover, caHIV-1 RNA can be measured on unstimulated cells or cells stimulated with T cell activating agents (inducible reservoir).

<u>HIV-1 RNA detection by PCR</u>

caHIV-1 RNA can be measured from RNA extracts of different cell types, usually CD4+ T lymphocytes, by RT-qPCR or RT-ddPCR. Targets of caHIV-1 RNA include primer/probe for us-RNA (against *gag, pol*, and LTR) [144–146], multiple spliced (ms-RNA; against a region containing *tat/rev* or *tat/nef* exon-exon junction)[144,145,147], polyadenylated transcripts[148,149], TAR RNAs[150], and the chimeric host-HIV read-through transcripts[149,151].

Other RNA PCR approach named tat/rev Induced Limiting Dilution Assay (**TILDA**) was developed to measure the frequency of peripheral CD4^{+} T cells expressing caHIV-1 RNA (Fig. 12). In this assay, CD4^{+} T cells are maximally stimulated with PMA/ionomycin to induce provirus expression for 12 hours and serially diluted to measure msRNA transcripts by RT-qPCR[152]. TILDA measures tat/rev transcripts that are required, but not enough, to produce viral particles. Also, as tat/rev transcripts are generated after splicing, it reduces the detection of defective proviruses.
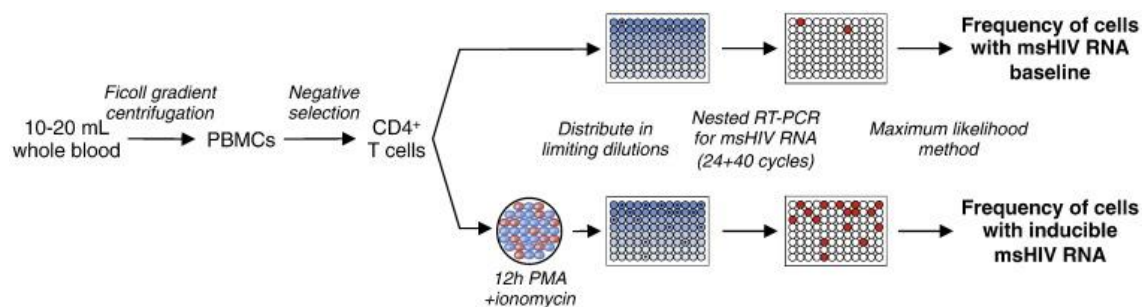
Figure 12. Schematic representation of TILDA. PBMCs are isolated from 10-20 ml of blood and enriched in CD4^{+} T cells by magnetic negative selection. CD4^{+} T cells are stimulated for 12 hours with PMA/ionomycin or resting. Then tat/rev transcripts are measured by a nested RT-PCR, and the frequency of inducible msRNA is determined using maximum likelihood method. Reproduced from Procopio FA. *et al*[152] .

HIV-1 RNA staining

Alternative methods have been developed to measure the transcription-competent reservoir able to produce RNA. These techniques combine fluorescence in situ hybridization (FISH) technique and branched double stranded DNA signal amplification, which is normally coupled with concurrent antibody staining for phenotypic markers. These assays allow the detection of single HIV-1 RNA positive cells and can be visualize by microcopy or flow cytometry, which have the main advantage that can localize what type of cells are producing virus.

The microscope technique, known as **RNAscope** (Fig. 13)[153,154], is based on a series of DNA probes with two sections, one recognizes the mRNA and the other forms part of a conserved tail sequence. Pairs of probes which recognizes adjacent regions of the target mRNA contain one half of the conserved tail, and when combined can be recognized by a DNA pre-amplifier which is in turn recognized by a secondary amplifier. This amplified structure is then labeled with a fluorescent probe, an alkaline phosphatase or horseradish peroxidase molecule that can be

visualized by a microscope. The benefit of this technique is that it preserves the tissue structure, facilitating to understand the types of cells and anatomic structures in which the virus is produced and how it is stored. Moreover, a novel multiplex microscopy ISH approach has been recently developed to simultaneously visualize vRNA, vDNA and protein in the same tissue section and therefore identify transcriptionally latent infected cells.
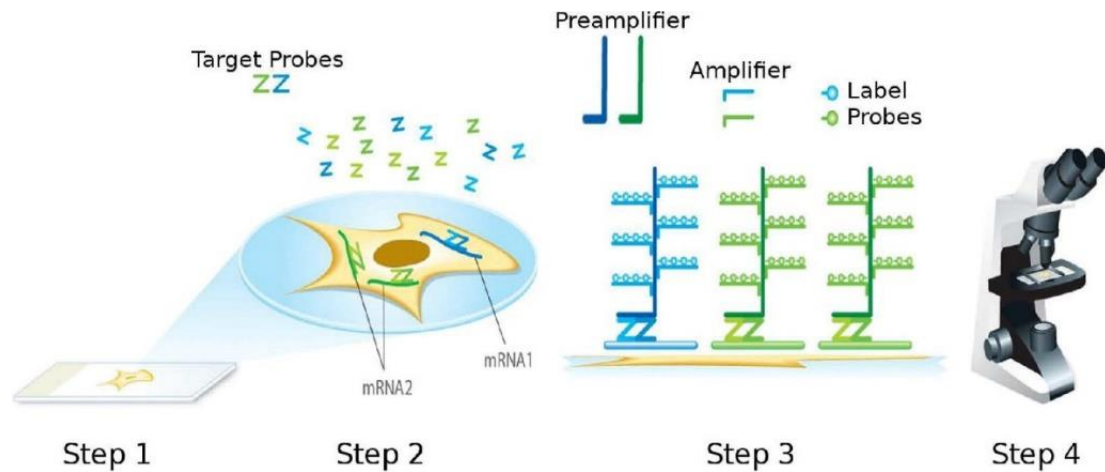
Figure 13. Schematic representation of the RNAscope assay procedure. First, cells or tissues are fixed and permeabilized to allow for target probe access. Then, target mRNA-specific probes (Z) are hybridized in pairs (ZZ) to multiple RNA targets. Multiple signal amplification molecules are hybridized, each recognizing a specific target probe, and each unique label probe is conjugated to a different fluorophore or enzyme. Finally, signals are detected using a microscope. Reproduced from Wang F. *et al*[155].

Regarding the flow cytometry techniques, different assays have been developed including a commercial assay named **PrimeFlow**™ or the **RNA FISH-Flow**[156–158]. These assays are basically based on the reactivation of cells with phytohemagglutinin (PHA) and ionomycin, and then stained by FISH using probes targeting the HIV-1 RNA *gag-pol* regions (Fig. 14). These techniques apart from the single cell phenotyping, have the advantage that are high throughput. However, are labor intensive and required a high number of starting cell input (Table 1).
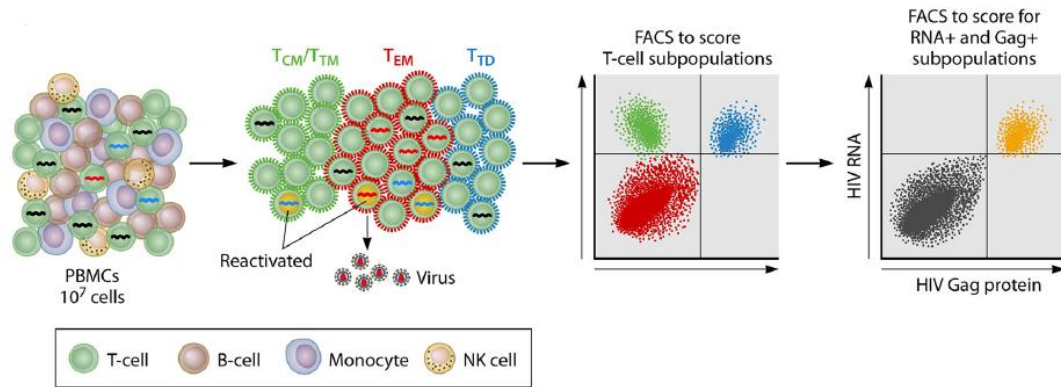
Figure 14. Schematic representation of the RNA FISH-Flow assay. PBMCs are use directly or after reactivation. Then, HIV-1 RNA and Gag protein as well as surface markers, are analyzed by flow cytometry. Reproduced from Prasad VR. *et al*[159].

1.5.3.4. Translation-competent reservoir

The previous assays described measure the transcription-competent reservoirs expressing HIV-1 RNA, but these are not necessarily able to produce infectious virions or even HIV-1 proteins. Therefore, assays able to quantify baseline or inducible expression of viral proteins have been of great interest to identify the translation-competent cellular reservoir. Provirus capable of translating HIV-1 proteins are more likely to be replication-competent, since they are less likely to have major defects[160]. However, it has also been described that some defective proviruses are still capable of producing HIV-1 proteins[161].

The **PrimeFlow**[TM] or the **RNA FISH-Flow** assays previously described can be combined with the use of antibodies against the p24 capsid protein in whole cells in order to assess at the same time HIV-1 transcription and translation information within a single cell[156–158,162] (Fig. 15). Generally, they are limited by a false positive rate from non-specific antibody binding, which might be overcome by the using of two p24 antibodies[162].
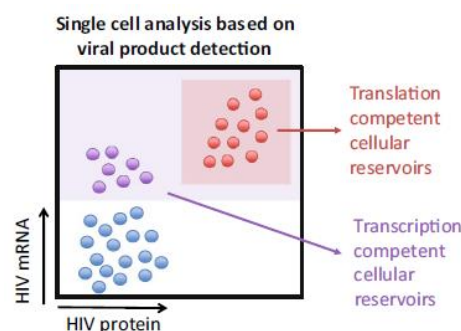
Figure 15. Single-cell strategy combining the detection of HIV-1 RNA and p24 production for the identification of the transcription and translation-competent reservoir by flow cytometry. Reproduced from Baxter AE. *et al*[163].

Other assay to measure viral proteins use the **digital enzyme-linked immunosorbent assay (ELISA) technology**[164] to quantify levels of p24 produced from cells after stimulation or in basal conditions. This technique is based on the capture of p24 molecules in solution by antibody loaded magnetic beads on an antibody immobilized solid phase. First, the p24 protein is captured by magnetic beads that have been coupled to an antibody to the target protein. Then, the captured p24 protein is labeled with a detection biotinylated antibody which is recognized by streptavidin-β-galactosidase. Finally, the beads are suspended in a solution containing a fluorogenic substrate to the streptavidin-β-galactosidase and loaded in femtoliters-size wells, termed as single-molecule arrays (SIMOA), returning a digital signal for analysis (Fig. 16). This technology can be applied to cell lysate or culture supernatants, being able to detect sub-femtomolar levels of p24.
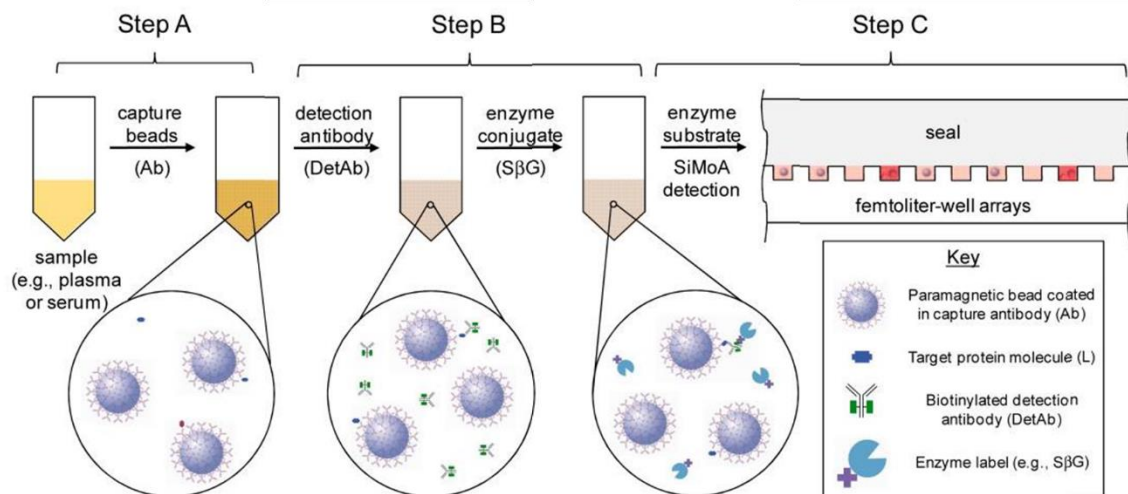
Figure 16. Schematic representation of the Digital ELISA. Reproduced from Lei C. *et al*[165].

1.5.4.5. Replication-competent provirus

Finally, replication-competent proviruses are those capable to transcribe, translate and produce new mature virions. The assay able to quantify them is the quantitative viral outgrowth assay (**qVOA**), which measures the functional reservoir and has been considered the gold standard for determining the frequency of CD4$^+$ T cells harboring replication competent-proviruses since its development in the 1990s[166]. However, this assay underestimates the size of the reservoir due to suboptimal induction and/or inadequate propagation of all replication-competent viruses *in vitro* (Table 1)[132].

The qVOA consists of a limiting dilution of CD4$^+$ T cells, either total or resting, that are maximally stimulated with a T-cell-activating mitogen (usually PHA) in the presence of irradiated allogeneic PBMCs, to initiate HIV-1 transcription and replication. To expand the number of virions, the cells are co-cultured with CD4$^+$ T cells from an HIV-negative donor, a reporter cell line or CD8-depleted PBMCs from three HIV-negative donors activated with three different stimulus (dubbed 3×3 cells) or MOLT-4/*CCR5* cells. After 14 days, viral outgrowth is assessed by an ELISA for the presence of p24^{Gag} in the culture supernatant. Poisson statistics are applied to estimate the frequency of replication-competent provirus that is reported as infectious units per million cells (IUPM) (Fig. 17).
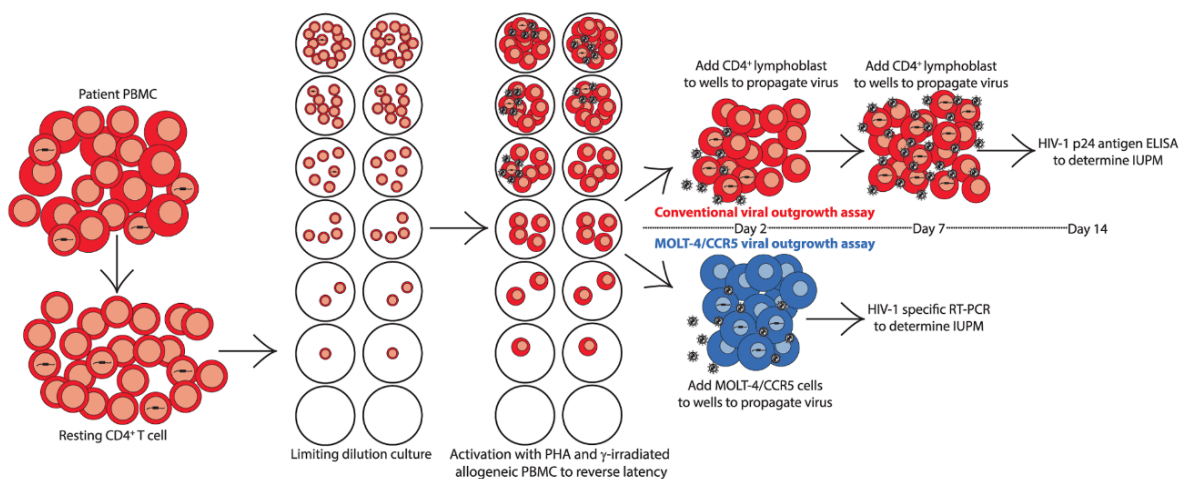
Figure 17. Schematic representation of the steps of the viral outgrowth assay (qVOA). Reproduced from Laird GM. *et al*[167].

A modification of the qVOA is the murine Viral Outgrowth Assay (**mVOA**)[168]. In this assay, CD4$^+$ T cells or PBMCs from HIV-1-infected individuals are injected into humanized immunocompromised mice (NSG mouse). The human cells will become activated in the mouse, releasing virus into circulation. The virus released is monitored in murine plasma by HIV-1 specific RT-qPCR. mVOA is not quantitative but gives a more physiological vision of what could happen if therapy is interrupted. Therefore, this method may have utility when *ex vivo* assays cannot detect latent infection (Fig. 18).
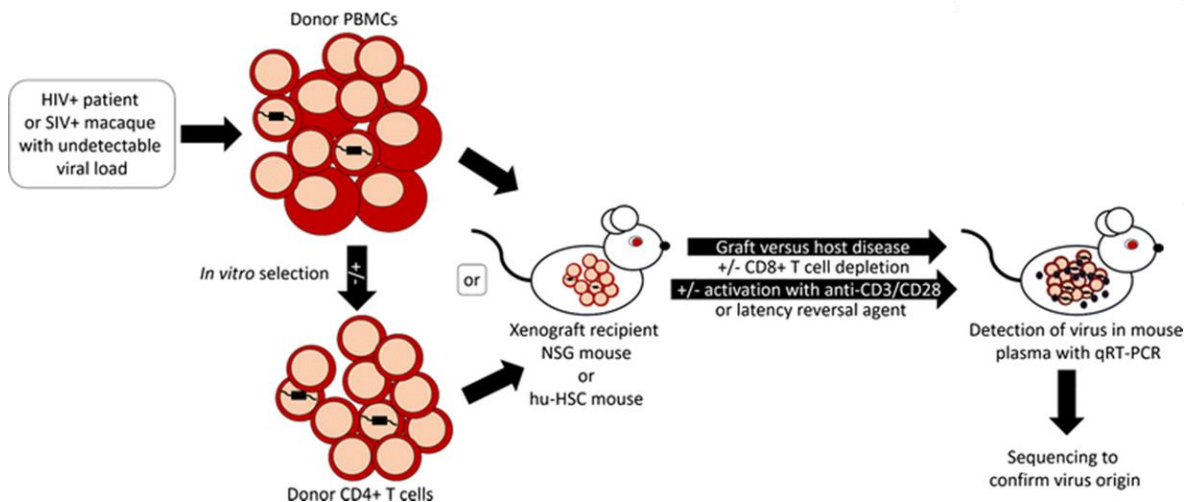
Figure 18. Schematic representation of the mice viral outgrowth assay (mVOA). Modified from Kelly A. *et al*[169].

1.6. The search of a cure

As previously stated, cART is not able to eradicate HIV-1 due to the presence of latently infected cells. The need of life-long treatment is associated with adverse effects, social stigma, persistent inflammation, and a constant threat of resistance emergement[170]. Therefore, a cure for HIV-1 infection is a major goal of research. There are two major concepts for HIV-1 cure, a sterilizing and a functional cure. A **sterilizing cure** would be achieved by a complete elimination of the HIV-1 virus from the body while a **functional cure** seeks to use the immune system to keep the virus at an undetectable level without the need of antiretroviral therapy.

1.6.1. HIV-1 cure strategies

Several approaches are being pursued including treatment optimization, latency reversal, latency silencing, immunotherapy, HIV-1 specific immune enhancement, and gene or cell therapy.

1.6.1.1. Treatment optimization

One of the proposed strategies to cure HIV-1 is based on treatment optimization with either intensification of treatment or early treatment initiation.

The **intensification of treatment** is based on the presence of low-level viral replication during cART, which may be due to a partial suppression or suboptimal drug concentrations in some tissues. Therefore, treatment intensification aims at

targeting the source of residual replication by adding additional drugs to accelerate the decay of the latent reservoir, leading in some individuals eventually to a cure. Several studies have been performed with compounds such as raltegravir[141], maraviroc[171], or darunavir/ritonavir[172] but with limited evidence on reducing the latent reservoir or residual plasma viremia. Nonetheless, a recent study with ABX464 has shown promising results showing a reduction in the viral reservoir and transcription initiation in suppressed individuals[173].

On the other hand, **treatment on early infection** has been shown that leads to a lower viral reservoir in peripheral blood and tissues, less immune activation, better immune reconstitution, and lower risk of serious AIDS-related events[174–176]. Moreover, different studies have shown that the small proportion of the individuals that are able to control viremia when therapy is interrupted, known as post-treatment controllers, started cART early after infection[72,177]. Also, this effect is shown in pediatric HIV-1 cases, including the "Mississippi baby"[178]. This newborn was infected intra-utero and treated 30 hours after birth, continuing the treatment until 18 months of age when therapy was interrupted. The baby remained undetectable for 27 months, when unfortunately the virus rebounded and the treatment had to be reinitiated.

1.6.1.2. Latency reversal

One of the most studied eradication approaches is the named as "**shock and kill**". This aims to combine an effect at reversing the transcriptional silencing of the integrated provirus through latency reversal agents (LRA) (shock) with the elimination of the new virions through the combined effects of cART and/or HIV-1 specific immune responses (kill) (Fig. 19).
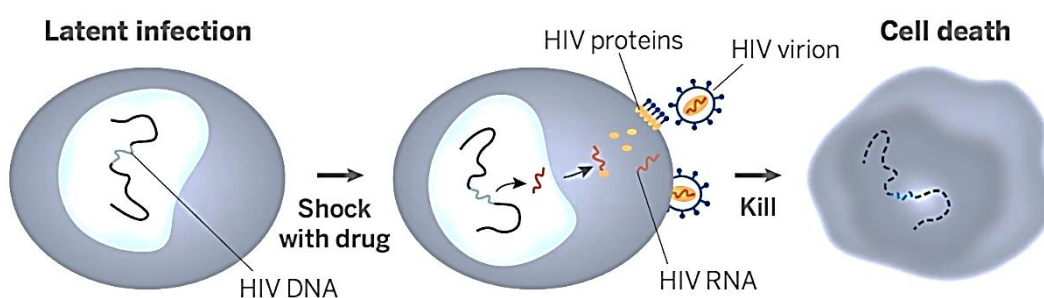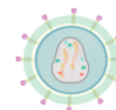
Figure 19. Schematic representation of the shock and kill strategy to eliminate HIV-1 latently infected cells. Reproduced from Cohen J. *et al*[179]

Multiple classes of LRA have been identified that successfully induced RNA production, but only some drugs induced the production of proteins and viral particles. These include histone deacetylase and histone methyltransferases inhibitors that upregulate the transcription by reversing epigenetic silencing[180–182]; protein kinase C agonists[183,184] and CCR5 agonists[185,186] that stimulate latent HIV-1 by activating NF-кb.

However, none of these interventions were capable of reducing the viral reservoirs, probably due to the limited ability of the compounds to reverse the latency and/or because the immune system needed to be primed to clear antigen-expressing cells[187].

A second generation of LRA seem to have promising results in pre-clinical studies such as the *small-molecule inhibitor of apoptosis antagonists* known as SMAC mimetic compounds (Second mitochondria-derived activator of caspases), that have demonstrated potent activity in different animal models by reversing HIV-1 latency[188]. Similarly, a study demonstrated that the FDA-approved retinoic acid derivative, known as acitretin, reactivate latent HIV-1 and induced preferential apoptosis of HIV-1 latently infected cells *in vitro*[189]. Also, toll-like receptors (TLR) agonists have shown latency reversing activity in non-human primates[190–192].

Finally, to do the "kill", it has been proposed the use of immunotherapies or therapeutic vaccinations, which will be explained in the following sections. In fact, a recent study in non-human primates has shown that the combination of a TLR-7 agonist with broadly neutralizing antibodies induced a state of remission[193].

1.6.1.3. Latency silencing

Since the shock and kill strategy has not been proven successful yet, an opposite approach has been proposed, termed as block and lock. This strategy aims to permanently silence the provirus, even in the absence of cART, with the use of latency-promoting agents (LPA). These drugs target HIV-1 transcription related viral and cellular factors to silence HIV-1 transcription in latent HIV-1 infected cells.

To date, the most advanced study involves the use of didehydro-cortistatin A (dCA), an inhibitor of the viral transcriptional activator Tat. *In vivo*, dCA has shown to block HIV-1 transcription and prevent viral reactivation. In humanized mice, dCA
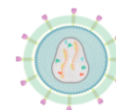
administration during cART has shown a modest delayed and reduced viral rebound upon treatment interruption[194]. This approach, still pending to be confirmed in humans, is a proof of concept of functional cure.

Other promising candidates for LPA include the use of the integration inhibitor LEDGINs, mTOR inhibitors, kinase inhibitors, and Jak-STAT inhibitors, among others (reviewed in [195]). Nevertheless, these strategies are in early stages of development.

1.6.1.4. Immunotherapy

Constant antigenic stimulation during HIV-1 infection leads to chronic immune activation, inflammation, and immune exhaustion. In this context, HIV-1 specific cells diminish or become dysfunctional, losing their antiviral and proliferative capacity to eliminate productively infected cells. Therefore, immunotherapy strategies are aimed at reversing the immune exhaustion to enhance anti-HIV-1 immune responses, principally by using immune checkpoint blockers.

Immune checkpoint blockers are antibodies against immune checkpoint molecules such as PD-1 or CTLA-4. It has been shown an enrichment for HIV-1 provirus in CD4$^+$ T cells expressing immune checkpoint molecules, and also a high expression of these molecules on HIV-1 specific CD8$^+$ T cells. Therefore, these antibodies have the potential to boost T-cell function and to act as an LRA. *Ex vivo*, antibodies against immune checkpoint molecules have demonstrated an enhancement on HIV-1 specific CD8$^+$ T cell responses[196,197] and viral production by CD4$^+$ T cells[198,199]. Different studies have suggested that immune checkpoint blockers are safe and efficacious in HIV-1-infected individuals with advanced stage cancers[200–204]. However, the effectiveness of immune checkpoint blockers to boost the immune system or eliminate the viral reservoir in HIV-1-infected individuals is still controversial. Results from studies using different immune checkpoint blockers range from showing no changes in HIV-1 specific-CD8$^+$ T-cell responses or HIV-1 reservoir[205], transient enhancement of HIV-1 specific-CD8$^+$ T cells with no variation in viral persistence[206,207], transient increase in viral production without changes in viral reservoirs[208,209], or cases of depletion of the HIV-1 reservoir[199,210]. Moreover, it has been recently published that in non-human primates the combination of PD-1/CTLA-4 blockade induces a robust latency reversal and reduction of integrated

virus but insufficient to achieve viral control[211]. Altogether, it seems that immune check point blockade is unlikely to induce HIV-1 remission in the absence of additional interventions.

1.6.1.5. HIV-1 specific immune enhancement

To enhance HIV-1 specific immunity, it is being developed the use therapeutic vaccination, broadly neutralizing antibodies (bNAbs) and chimeric antigen receptor T cells. These strategies are aimed at eliminating or significantly reduce viral rebound when therapy is interrupted by enhancing the host immune response to HIV-1, thus achieving a functional cure.

Therapeutic vaccination aims to boost the magnitude, breath of antigen-specificities and functionality of anti-HIV-1 T-cell responses to eliminate or control HIV-1 infected cells in the absence of cART. In therapeutic vaccine trials, the vaccine is administered during cART, followed by a period of cART interruption to assess efficacy by time to viral rebound, size of the reservoir and host immune responses. Different vaccine strategies have been tried including live attenuated virus, death whole virus, replicating viral vectors, replication-deficient viral vectors, viral-like particles, soluble proteins, peptides and naked DNA, having different immunogenicity and safety[212]. Some of these vaccine strategies have shown an improvement of autologous HIV-1 specific T-cell responses but with limited success on viral control[213,214].

Recently, several studies have assessed the combination of an LRA with a therapeutic vaccine. For instance, the use of a vaccine and the LRA romidepsin led to a reduction in total HIV-1 DNA in one study[215], and viremic control after cART cessation in some participants from another study[216]. A recent report has shown a progressive decrease in the reservoir and recovery of the immune function after a Tat based immunization[217]. Moreover, in non-human primates other vectors that stimulate HIV-1 specific T cells look promising, including cytomegalovirus and the combination of an adenovirus vector with a TLR-7 agonist[218].

bNAbs can neutralize a wide range of viral strains, enhance CD8$^+$ T-cell function, and mediate different effector functions such as ADCC (antibody-dependent cellular toxicity), ADCP (antibody-dependent cellular phagocytosis),

ADCDC (antibody-dependent complement-dependent cytotoxicity) or ADCT (antibody-dependent trogocytosis). In non-human primates, the use of the monoclonal antibody PGT121 resulted in a decline of plasma viremia and reduced proviral DNA in peripheral blood, gastrointestinal mucosa and lymph nodes[219]. Furthermore, the early administration of VRC07-523 and PGT121 to 1 month-old non-human primates resulted in complete clearance of SHIV-infected cells[220]. In clinical trials with HIV-1-infected individuals, the combination of 3BNC177 and 10-1074 showed an effective suppression of viral rebound for a median of 21 weeks[221], with the prevention of viral replication in some individuals when cART is interrupted and boost of the CD8 cytotoxic activity[222]. Alternatively, the use of synthetic molecules that mimic antibodies such as eCD4-Ig, which mimic both CD4 and CCR5 receptors, have demonstrated to protect from SIV and SHIV infection in non-human primates[223,224].

Chimeric antigen receptors T cells (CAR-T) are autologous T cells genetically engineered that comprise an extracellular domain (derived from the CD4 receptor or anti-HIV-1 antibodies) that recognizes an HIV-1 epitope linked to an intracellular T cell receptor domain that induces a cytotoxic T lymphocyte response upon antigen binding. Thus, when re-administered to the individual can direct the cytotoxic response to cells expressing the disease epitope. *In vitro* studies have demonstrated virus-clearing using anti-HIV-1 CAR-T cells[225–227]. Also, CAR-T cells targeting multiple sites named as duoCAR have shown in humanized mice a potent antiviral activity and elimination of HIV-1 infected cells[228].

1.6.1.6. Gene and cell therapy

Finally, gene and cell therapy strategies aim to replace HIV-1 infected cells with a new virus-resistant hematopoietic stem or progenitor cells, and, therefore, generating an HIV-resistant immune system. The virus-resistant cells can be either derived from a natural resistant donor, by allogeneic hematopoietic stem cell transplantation (allo-HSCT), or by genome editing HIV-1 infected cells from the individual. Usually, these strategies are focused on mimicking the 32-bp deletion in the *CCR5* gene that confers natural resistance to the R5 viral strains present in some individuals.

Allo-HSCT, is the only eradication strategy that has achieved the HIV-1 remission or cure. In 2007, the "Berlin patient" who suffered from acute myeloid leukemia, received a myeloablative conditioning, two sessions of total body irradiation and two allogeneic stem cell transplants from a donor who was homozygous for the *CCR5* Δ32 mutation[229]. The individual stopped cART at the time of the transplant and remain undetectable for HIV-1 for more than 10 years, until September 2020 when he died of leukemia relapse. Also, no HIV-1 RNA or HIV-1 DNA were detectable in peripheral blood, BM or GALT, and thus it is considered that achieved a sterilizing cure[230]. This case encouraged investigators to replicate the strategy in other individuals such as the "Boston patients"[231] using a *CCR5* wild-type donor, achieving a delayed viral rebound when therapy was interrupted but not a sterilizing cure. Additionally, the consortium named IciStem has been extensively following HIV-1-infected individuals who undergo allo-HSCT for medical reasons. As part of IciStem, two more cases of persistent HIV-1 remission after an allo-HSCT with *CCR5* Δ32 cells have been achieved, which are known as the London[232,233] and Düsseldorf[234] patients.

On the other hand**, gene therapy** aims at directly modify a specific sequence of DNA by genome-editing techniques such as zinc fingers nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats/CRISP-associated protein nuclease-9 (CRISP/Cas9). The approach would involve the disruption of the *CCR5* gene in autologous CD4$^+$ T cells or hematopoietic stem cells followed by the reinfusion of the modified cells, with the aim of mimic the natural *CCR5* Δ32 mutation to make the cells resistant to new infections. In 2014, a clinical trial using ZFNs showed that the modification of the *CCR5* gene was safe but it did not prevent from viral rebound once therapy was interrupted in all the participants[235]. New gene strategies are under current investigation involving the modification of stem cells, different conditioning regimens or using other genome-editing tools such as CRISPR/Cas9. Alternatively, gene editing may be used to knockout or attenuate the HIV-1 provirus by targeting the LTR or *gag* genes to disrupt viral gene expression or cleave it out entirely from the cell genome[236,237].

1.7. Thesis rational

As previously stated in this introduction, it exits different individuals with a natural control of the HIV-1 infection as EC[238] and PTC[72], which are characterized for having a small size HIV-1 reservoir. On the other hand, the wide variety of eradication strategies under research are seeking to reduce the HIV-1 reservoirs to prove their effectiveness. Thus, having or achieving low levels of HIV-1 reservoir seems to be key for any HIV-1 cure strategy and the final goal is to eradicate the infection. However, there are many unresolved questions such as the clinical, viral, and immunogenetic factors associated with the reduction of the HIV-1 reservoir and its implication for achieving a functional or sterilizing cure. Those questions will be approached in this thesis in three different groups of HIV-1-infected individuals.

First, it will be evaluated a group of exceptional EC (EEC) that have maintained a sustained control of HIV-1 replication and low HIV-1 reservoirs for more than 25 years in the absence of antiretroviral treatment. These individuals may represent cases of spontaneous functional cure, what would prove the importance of limiting the reservoir burden for long time. The factors associated to this extremely long control are unknown and have been addressed in the chapter 3a of this thesis, and published as: "Permanent control of HIV-1 pathogenesis in exceptional elite controllers: a model of spontaneous cure (*Scientific Reports*, 2020)".

However, low levels of HIV-1 reservoir are not only associated to the exceptional cases of HIV-1 control. There are individuals that also harbor low levels of HIV-1 reservoir but are not able to control the viral replication by themselves in the absence of cART[239–242]. It is known that individuals who start cART in the early stages of infection, especially in Fiebig I, have a limited reservoir[68]. Nonetheless, it seems that early initiation of cART would not be the only factor associated with having a low reservoir since it has been described individuals starting cART on the chronic phase of the infection with similar characteristics[239,243,244]. Knowing the kinetics of the reservoir establishment, as well as the immunological and virological factors in periphery and anatomic sanctuaries associated with the spontaneous reduction of the HIV-1 reservoir, without eradication interventions, in those individuals is key to later design more effective eradication strategies. This has been addressed in

chapters 3b and 3c of this thesis and published as: "Extremely Low Viral Reservoir in chronically treated HIV-1-infected individuals (*EbioMedicine*, 2020)".

After the characterization of two different models of spontaneous reductions of the HIV-1 reservoirs, it is important to contrast the results with an intervention that can achieve the same outcome. So far, the only cure strategy capable of attain this objective is the hematopoietic stem cell transplant (HSCT), as previously mentioned. This strategy not only was able to reduce the viral reservoir but also to achieve a persistent HIV-1 remission when cART was interrupted in specific cases of transplantation with a *CCR5* Δ32 donor. However, the intrinsic factors related to the transplant that help to reduce the viral reservoir are still unknown and might be of high importance to try to develop less invasive strategies to apply to a broader population of HIV-1-infected individuals. This was addressed in chapter 3d of the thesis and published as: "Mechanisms That Contribute to a Profound Reduction of the HIV-1 Reservoir After Allogeneic Stem Cell Transplant (*Annals of Internal Medicine*, 2019)".

Altogether, this work will be a proof of concept of the ability of different approaches to reduce the HIV-1 reservoirs, obtaining key information to design better tools for a future HIV-1 cure.

CHAPTER 2. HYPOTHESIS AND OBJECTIVES

We **hypothesize** that exist different mechanisms that lead to a reduction in the HIV-1 reservoir to extremely low or undetectable levels, and those vary when we observe a spontaneous decrease or when using eradication strategies. This reservoir decrease would be the first step required to achieve an HIV-1 cure, although it might be necessary the interaction with additional strategies to reach to a sterilizing cure.

The **global aim** is to characterize the clinical, viral and immunogenetic factors associated with the reduction of the HIV-1 reservoir in three different models: natural control of the infection (Exceptional Elite Controllers), a natural low HIV-1 reservoir despite regular HIV-1 progression and being under cART (LoViReT), and after an aggressive intervention that reduce the HIV-1 reservoirs (allo-HSCT). The factors analyzed will be related to their putative role in the HIV-1 eradication.

Objective 1: To deeply characterize clinical, virological, and immunological factors associated with natural HIV-1 control for more than 25 years in the absence of cART. This objective will be addressed in Chapter 3a, entitled "Permanent control of HIV-1 pathogenesis in exceptional elite controllers: a model of spontaneous cure".

Objective 2: To determine the clinical, virological, and immunological factors related to a reduction of the HIV-1 reservoir in a cohort of LoViReT (Low Viral Reservoir treated) individuals whom harbor an unusual extremely low viral reservoir, but without control of HIV-1 in the absence of cART. Those factors will be related to the longitudinal dynamics of the HIV-1 reservoir and the immune cells before and after initiation of cART between chronic treated LoViReT and controls. This objective will be addressed in Chapter 3b, entitled "Extremely Low Viral Reservoir in chronically treated HIV-1-infected individuals".

Objective 3: To deeply characterize the host immunogenetic and viral factors associated with the LoViReT cohort, including the replication-competence of the virus, and distribution among anatomical and cellular compartments. This objective will be addressed in Chapter 3c, entitled "LoViReT individuals show altered HIV-1 latency distribution".
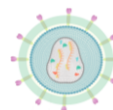
Objective 4: To evaluate the factors associated with the reduction of the viral reservoirs after an allogeneic hematopoietic stem cell transplant, specially using *CCR5* wild-type donors. This objective will be addressed in Chapter 3d, entitled "Mechanisms that contribute to a profound reduction of the HIV-1 reservoir after allogeneic stem cell transplant".

CHAPTER 3. RESULTS

Chapter 3a. Permanent control of HIV-1 pathogenesis in exceptional elite controllers: a model of spontaneous cure

This chapter corresponds to the manuscript:

<u>Author's contributions</u>: The author of this thesis contributed to the current work by testing individuals' samples and analyzing results from the total HIV-1 DNA, quantitative viral outgrowth assay, caHIV-1 RNA, ultrasensitive viral load, inhibition viral assay and humoral immune responses. The author also contributed designing the experiments, making the figures, and writing of the manuscript.

3a.0. PRESENTATION

The first chapter of this thesis includes the comprehensively study of three HIV-1-infected individuals, whom in the absence of antiretroviral treatment have been able to control HIV-1 replication for more than 25 years. These individuals represent cases of spontaneous functional cure, and therefore are of highly interest to study host and viral factors associated with their viral control.

3a.1. INTRODUCTION

Functional cure is defined as the permanent suppression of HIV-1 viral replication in the absence of cART even if full viral eradication is not achieved[245]. Elite controllers represent a small proportion of HIV-1-infected individuals with a spontaneous control of HIV-1 replication at undetectable levels. However, they are heterogeneous in terms of long-term clinical, virological and immunological progression. Among them, there is a subgroup of individuals who have an asymptomatic HIV-1 infection and prolonged control of clinical progression without cART. The closest cases of spontaneous functional HIV-1 cure are potentially represented by this clinical phenotype of slow or no disease progression[246]. The underlying mechanisms contributing to this control include host and viral factors[247,248]. Previous works have stablished differences in EC with long-term non-progression compared to EC that ultimately lost control[249]. Other reports have described several selected cases of durable control over HIV-1 replication without cART[71,250]; however, the cases described herein are Exceptional Elite Controllers (EEC) with no disease progression for more than 25 years. The question whether these individuals can be considered cured remains unresolved. Also, there is a need for differential markers that can characterize these unique cases of potential HIV-1 remission.

In this study, we characterized three EEC diagnosed for 25, 28 and 29 years with an extensive follow-up, permanent control of viral replication, and no sign of disease progression. We investigated these individuals to determine if they can be considered spontaneous cases of HIV-1 functional cure. Multiple host genetic, immunological, and virological factors were examined to distinguish this extraordinary phenotype and to identify potential diagnostic markers.

3a.2. METHODS

3a.2.1. Individuals, samples, and clinical characteristics
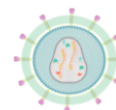
The study individuals were selected from a previous long-term EC cohort[248]. These individuals have been followed in the Centro Sanitario Sandoval (a primary sexual clinic without antiretroviral prescription belonging to the Hospital Clínico San Carlos. IdISSC, Madrid) for more than 20 years. All individuals were diagnosed with a second-generation ELISA (Abbott) and confirmed by Western-Blot (BioRad) after 5 to 6 years of the estimated infection. Participants gave informed consent for molecular and genetic studies. In March 2017, 500 ml of peripheral blood was drawn to perform a comprehensive analysis of multiple immunological and virological markers. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Research Ethics and Animal Welfare Committee of the Instituto de Salud Carlos III (CEI PI 05_2010-v3) and by the Ethics and Clinical Research Committee of the Hospital Clínico San Carlos in Madrid (Number C.I. 16/490-E).

3a.2.2. PBMCs isolation and preservation

Peripheral blood samples were processed to separate plasma from cell fraction. Plasma samples were stored at -80ºC and PBMCs were isolated by a standard Ficoll-Hypaque (Lymphoprep, Axis Shield Poc As™) density gradient centrifugation (390xg, 30 min.). Part of the PBMCs were used for fresh experiments and the rest aliquoted in cryovials (10-15 million PBMCs per vial), placed in a CoolCell® freezing container (Corning) at -80ºC overnight, and stored in liquid nitrogen until use.

3a.2.3. CD4$^+$ T-cell purification

CD4$^+$ T cells were isolated from fresh or thawed PBMCs by negative immunomagnetic selection (CD4$^+$ T cell isolation kit-human; Miltenyi Biotec) which is based on negative selection by cell labeling with biotinylated antibodies and subsequent incubation with magnetic microparticles coated with anti-biotin antibodies using the autoMACS® Pro Separator (Miltenyi Biotec), following the manufacturer's instructions. Briefly, for each 10^6 PBMCs, cells were resuspended in 4 µl of washing buffer (phosphate-buffered saline (PBS; Gibco, Invitrogen), 2 mM UltraPure® ethylenediaminetetraacetic acid (EDTA; Invitrogen, Life Technologies)

and 1% fetal bovine serum (FBS; Gibco, Invitrogen)) and 1 μl of CD4⁺ T cell biotin-antibody cocktail (α-CD8, α-CD14, α-CD15, α-CD16, α-CD19, α-CD36, α-CD56, α-CD123, α-TCRγ/δ, and α-CD235a); incubated at 4ºC for 5 minutes. Then, it was resuspended in 3 μl of washing buffer and 1 μl of CD4⁺ T cell microbead cocktail (microbeads conjugated to monoclonal antibodies against biotin and CD61), incubated at 4ºC for 10 minutes, and resuspended in 1 ml of washing buffer. After negative immunomagnetic selection, CD4⁺ T cells were counted with perfect-count microspheres™ (Cytognos), following the manufacturer's instructions, using the cytometer BD FACSCalibur™ (Becton Dickinson Biosciences).

3a.2.4. Viral persistence

3a.2.4.1. Total proviral HIV-1 DNA

Total proviral reservoir was quantified in two samples, one from 2017 and another 11 to 13 years before, by ddPCR[251]. Purified peripheral CD4⁺ T cells were resuspended in lysis buffer at a concentration of 5×10^4 cells/μl, which consisted of UltraPure® DNAse-RNAse–free water (Gibco, Invitrogen) containing 10 mM Tris-HCl (pH=9.0), 0.1% Triton x-100 (Sigma), and 400 μg/ml Proteinase K (Ambion). Cell extracts were incubated for 12-16 hours at 55ºC, then proteinase K was inactivated at 95ºC for 5 min. Lysed CD4⁺ T cells extracts were used to measure cell-associated total HIV-1 DNA, using two primer sets in the viral 5′ long terminal repeat (5'-LTR*)* and *gag* regions to circumvent sequence mismatches in viral sequences (Table 2). The Ribonuclease P/MRP Subunit P30 (*RPP30*) housekeeping gene (Table 2) was quantified in parallel to normalize sample input. Briefly, 10 μl of 2x ddPCR supermix for Residual DNA Quantification for *gag*/LTR primers and 2x ddPCR supermix for Probes (no Deoxyuridine-5'-triphosphate (dUTP)) (Bio-Rad) for *RPP30* was mixed with 1μl of 20x primers/probe mix (18 μM primers and 5 μM probe labelled in FAM or HEX (Integrated DNA Technologies), and 2 μl of lysed CD4⁺ T cells extract in a total volume of 20 μl. For *RPP30* quantification, the lysed CD4⁺ T cells extract was diluted 1:50 to avoid saturation of the signal. The mix reaction was loaded into a Bio-Rad droplet generator cartridge and 70 μl of droplet generation oil. The cartridge was placed in the droplet generator QX100™ device (Bio-Rad) where droplets were generated and transferred to a 96-well PCR plate, which was then sealed using PX1™ PCR plate sealer (Bio-Rad).

DNA was amplified using the C1000 Touch™ Thermal Cycler (Bio-Rad) (initial denaturation at 95ºC for 10 min; 40 cycles: 94ºC for 30 s and 57ºC for 60 s; final extension: 98ºC for 10 min; hold: 4ºC). After that, droplets were analyzed using a QX100™ droplet reader (Bio-Rad). Analysis of copies/µl of target DNA was performed using the QuantaSoft v.1.6 software (Bio-Rad, Hercules, CA, USA).

We analyzed 1.5–1.8 million cells in several replicates, with a LOD of 1–3 copies/10^6 CD4$^+$ T cells. PBMCs from HIV-negative donors were used as negative controls and assayed in each plate to set the positive/negative threshold. The number of those negative control wells was the same as that of the replicates for each sample.

Table 2. Primer and probe sequences

Name	Sequence (5'-3')	Strand	Target	Ref
LTR-U5 integrated	GTTCGGGCGCCACTGCTAG	Forward		
LTR-R integrated	TTAAGCCTCAATAAAGCTTGCC	Reverse	5'-LTR	[136]
New integrated-2 Probe	CCAGAGTCACACAACAGACGGGCA	Probe		
HIV_F (SCA)	CATGTTTTCAGCATTATCAGAAGGA	Forward		
HIV_R (SCA)	TGCTTGATGTCCCCCCACT	Reverse	*gag*	[118]
HIV Probe (SCA)	CCACCCCACAAGATTTAAACACCATGCTAA	Probe		
RPP30-F	GATTTGGACCTGCGAGCG	Forward		
RPP30-R	GCGGCTGTCTCCACAAGT	Reverse	*RPP30*	[252]
RPP30-Probe	CTGACCTGAAGGCTCT	Probe		
TBP-S	TTCGGAGAGTTCTGGGATTGTA	Forward		
TBP-AS	TGGACTGTTCTTCACTC	Reverse	*TBP*	[253]
TBP-Probe	TTGGCCCGTGGTTCGTGGCTCTCTTATCCTCA	Probe		

3a.2.4.2. Quantitative Viral Outgrowth Assay (qVOA)

The replication-competent reservoir was measured on fresh CD4$^+$ T cells from each individual in 28–63 × 10^6 CD4$^+$ T cells in a limiting dilution cell culture assay, as previously described with minor modifications[62]. Briefly, CD4$^+$ T cells from the individuals were cultured in serial dilutions ranging from 25×10^6 to 32 cells per well. CD4$^+$ T cells were co-cultured with irradiated allogeneic HIV-negative PBMCs in the presence of 2 µg/ml PHA (Sigma), interleukin-2 (IL-2, Novartis), and T-cell growth factors (TCGF). After 24 hours, PHA was removed, and CD8-depleted donor PBMCs previously activated under 3 different conditions (OKT3, 5 µg/ml of PHA and 0.5 µg/ml of PHA), known as 3×3 cells, were added to the co-culture as targets for viral infection[254,255]. Cell culture medium (Rowell Park Memorial Institute (RPMI)-

1640 (Gibco, Invitrogen) + 1% Penicillin/Streptomycin (Gibco, Invitrogen) + 10% FBS + 100 U/ml IL-2 + 2% TCGF) was changed at days 5 and 8, and freshly prepared 3×3 cells were added to the co-culture at day 8. Supernatants from day 14 were quantified with p24^{Gag} ELISA (Perkin-Elmer, USA) to identify positive wells. Infectious Units Per Million (IUPM) CD4$^+$ T cells were determined using IUPMStats v.1 (https://silicianolab.johnshopkins.edu/) based on the maximum likelihood method.

3a.2.4.3. Plasma viral load and residual viremia

Viral load was determined with the Quantiplex HIV-1 RNA 2.0, 3.0 and the Versant NA (Siemens, Germany) with different limits of detection. Residual viremia (HIV-1 RNA) was measured by ultracentrifugation in 9 ml of plasma at 170,000g at 4°C for 30 min, followed by viral RNA extraction using the m2000sp Abbott RealTime HIV-1 Assay device and laboratory-defined applications software from the instrument. HIV-1 RNA copies in the low range were determined by an in-house calibration curve set (range, 10–10^3 absolute copies)[119], which had previously been validated using a standard HIV-1 DNA control from the World Health Organization (WHO) in the range of 128–0.5 copies per ml. The LOD was 0.5 copies/ml in all the individuals.

3a.2.4.4. Cell-associated HIV-1 RNA

Viral transcription was evaluated by quantification of cell-associated HIV-1 RNA in purified CD4$^+$ T cells that were preserved in RNAlater solution (Ambion) until RNA extraction. RNA was extracted using RNeasy mini kit (Qiagen), following the manufacturer's instructions, and RNA concentration and quality were determined by NanoDrop spectrophotometer (ND-1000; Thermo Fisher Scientific). Purified RNA extracts were amplified by one-step reverse-transcription ddPCR (Bio-Rad) with primers and probe in the viral 5'-LTR and *gag* gene. The expression of the housekeeping gene TATA-binding protein (*TBP*) was quantified in parallel to normalize RNA input[253] (Table 2). Briefly, 5 µl of 2x One-Step RT-ddPCR Mastermix (Bio-Rad) was mixed with 300nM of Dithiothreitol (DTT), 1 µl of 20x primers/probe mix (18 µM primers and 5 µM probe (Integrated DNA Technologies)), 2 µl of reverse transcriptase enzyme and 8 µl of RNA sample in a total volume of 20 µl. The mix reaction was loaded into a Bio-Rad droplet generator cartridge and the droplets were

generated, as previously described. RNA was retro-transcribed and amplified using the C1000 Touch™ Thermal Cycler (Bio-Rad) (retro-transcription: 60ºC for 30 min; enzyme activation: 95ºC for 5 min; 40 cycles: 94ºC for 30s and 60ºC for 60s; final extension: 98ºC for 10 min; hold: 4ºC), and, after cycling, droplets were analyzed immediately using a QX100™ droplet reader (Bio-Rad). Analysis of copies/µl of 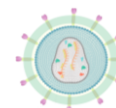target RNA was performed using the QuantaSoft v.1.6 software (Bio-Rad) and normalized using the copies/µl of *TBP* mRNA.

Samples were analyzed on several replicates with 3–5 million of CD4$^+$ T cells for each subject, with a variable LOD between 0.03 and 0.05 (ratio HIV/*TBP*).

3a.2.5. Genetic studies

3a.2.5.1. Nucleotide sequence and phylogenetic analysis in env gene

Sequences were obtained by limiting dilution in a 614 base pair (bp) C2–V5 fragment in *env* gene[256]. All sequences with gaps and hypermutation (only two in one sample from EEC-3) were excluded of analysis. Near full-length genome analysis was performed with overlapping limiting dilution PCR[132].

All nucleotide sequences generated in this study were submitted to GenBank under accession numbers: AY501160–AY501179, AY501254–AY501269, KC595083–KC595089, KC595099–KC595105, KC595118–KC595119, EU644051, EU644056–EU644060, MH595843, MH595844, MH595846, MN068093–MN068211 and MN055643.

3a.2.5.2. Quasispecies diversity analysis

We selected sequences from individual quasispecies in the HIV-1 database (http://hiv.lanl.gov/) from the same region analyzed in the study. We included infected individuals with undetectable viral load under prolonged cART (range [2–15 years])[257,258], LTNPs with more than 10 years of infection and transient EC[249] defined as individuals that lost spontaneous viral control during the follow-up, for comparison with the study individuals. We estimated the average diversity over sequence pairs for each time point in every individual and calculated the average number of base differences per site (p-distance) with 500 bootstrap replicates using MEGA6. All positions containing gaps and missing data were eliminated.
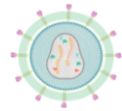
Differences in the average p-distance per individual between groups were tested for significance using pairwise Wilcoxon Rank-Sum Test in R v3.5.0, applying the Benjamini-Hochberg procedure for multiple comparisons correction (cut off for significance False Discovery Rate 0.05).

3a.2.6. Immune responses

PBMCs were thawed, washed and in vitro stimulated with overlapped HIV-1 (Gag)-specific peptide pool (NIH AIDS Research and Referenced Reagent Program) as previously described[249,259].

Stimulated PBMCs were stained with surface and intracellular marker antibodies (Fig. 20)[259]. PBMCs were analyzed in a LSR Fortessa Cell Analyzer (BD Biosciences, Spain).
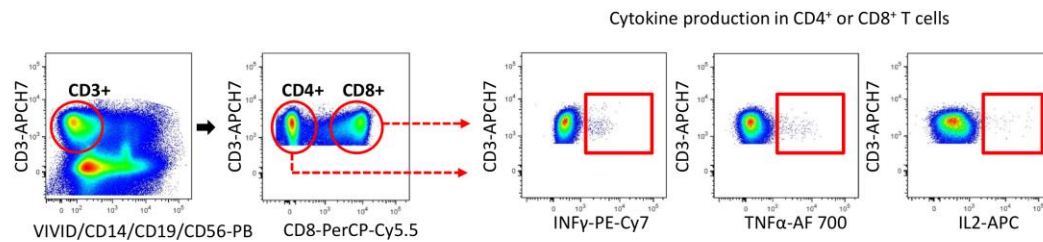
Figure 20. Schematic diagram of the flow cytometry gating strategy for (Gag-specific CD4+ and CD8+ T-cell. Representative plots show the functional cytokine response to Gag peptides.

3a.2.7. Susceptibility and viral inhibition assay

CD4+ T cells alone or in combination with autologous CD8+ T cells from each individual were infected by spinoculation with 40 nanograms of p24^{Gag} of the laboratory viral strain HIV-1$_{NFN-SX}$ (CCR5 tropic), and HIV-1$_{NL4-3}$ (CXCR4 tropic). CD4+ T cells were activated for 3 days with PHA and IL-2 before infection. Supernatants were sampled at day 7 and quantified with HIV-1 p24^{Gag} ELISA (Perkin-Elmer life Sciences).

3a.2.8. Assay of soluble biomarkers
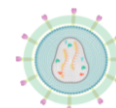
Plasma samples were collected in EDTA-lined tubes, and aliquoted and stored at −20°C. The levels of high sensitivity C-reactive protein (hsCRP) and β2-microglobulin (β2M), D-dimer, IL-6 and sCD163 were performed as previously described[260]. Specific HIV-1 antibodies were measured in plasma samples using a low-sensitive (LS) version of the VITROS anti-HIV-1 assay (Ortho-Clinical Diagnostics) and a limiting antigen avidity assay (Lag-Avidity) (Sedia), as previously

described[261]. Briefly, in the LS-VITROS four recombinant antigens (HIV-1 Env 13, HIV-1 Env 10, HIV-1 p24, and HIV-2 Env AL) derived from HIV-1 core, HIV-1 Env, and HIV-2 Env proteins were quantified using one part in 400 parts dilution of the plasma. The avidity assay measures the capacity of guanidine to elute low-avidity and low-affinity antibodies after antigen-antibody bonds have formed. The results are reported as an avidity index, calculated as the ratio of the signal to cut-off (S/CO) of the sample incubated in guanidine to the S/CO of the sample incubated in PBS.

3a.2.9. Statistical analysis

Changes over time in total HIV-1 DNA were evaluated using the Wilcoxon signed rank test. Gag-specific T-cell response poly-functionality was quantified with the poly-functionality index algorithm (pINDEX)[262] employing 0.1.2 beta version of the "FunkyCells Boolean Dataminer" software (www.FunkyCells.com) provided by Dr. Martin Larson (INSERM U1135). Analyses were performed with Prism 7 (GraphPad) and the statistical significance was set at 5% for all tests.

3a.3. RESULTS

3a.3.1. Clinical characteristics of the individuals

The three individuals of the study (EEC-3, EEC-9, and EEC- 56) maintained undetectable viral loads (except two early blips in EEC-9) and stable CD4$^+$ and CD8$^+$ T-cell counts in absence of cART for more than 25 years of infection (Fig. 21 a–c and Table 3). Hepatitis C Virus (HCV) infection was diagnosed in the three individuals because of intravenous drug use (IVDU) and all reported viral clearance, either spontaneously or after treatment (Table 3). The individuals reported no further exposure to HIV-1 since their diagnosis. The two females of the study gave birth uninfected children in absence of any cART. All individuals were classified as clinical status A, with no symptoms of HIV-1 clinical progression or AIDS defining events.
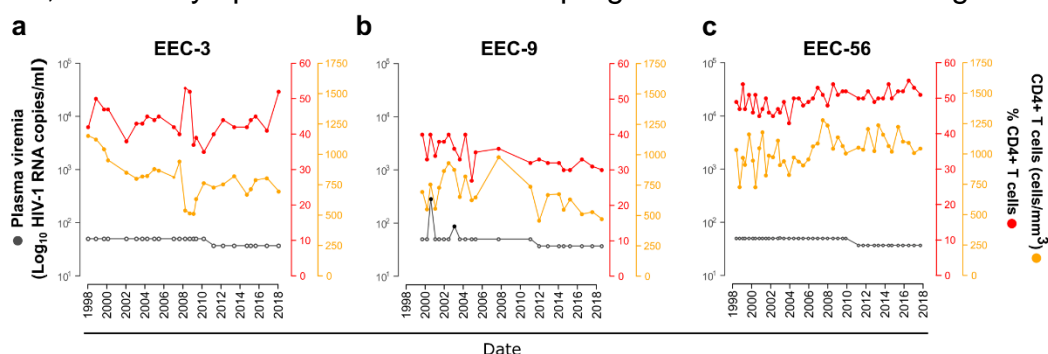
Figure 21. Clinical characteristics. (**a–c**) Plasma RNA viral load, absolute CD4$^+$ and CD4$^+$ T cell percentage over time in the individuals studied. Open symbols for viral load indicate values below the detection limit

Table 3. Clinical, epidemiological, and host-genetic characteristics

	EEC-3	EEC-9	EEC-56
Year of birth	1956	1957	1957
Sex	Male	Female	Female
Race	Caucasian	Caucasian	Caucasian
Year of HIV-1 diagnosis	1988	1992	1989
Age at diagnosis	32	35	32
Transmission route[a]	IVDU	IVDU	IVDU
Remarks	–	HIV-1 negative child	HIV-1 negative child
HCV coinfection	1996	1992	1993
Treatment	RBV/IFNα (2008)	Spontaneous clearance	DAA (2017)
Genetic markers			
CCR5 Δ32 rs333[b]	11	11	11
CCR2 V64I rs1799864[b]	11	11	11
HLA C rs9264942[b]	22	22	22
HLA A	02:01, 02:05	02:01, 31:01	01:01, 02:01
HLA B	27:05, 58:01	39:01, 57:01	14:02, 57:01
Genetic Score	4	3	4

[a]No further expositions after HIV-1 diagnosis; [b]"1" indicates the most frequent allele and "2" the mutant. IVDU= intravenous drug user; RBV= ribavirin; IFNα= interferon α, DAA= direct acting antivirals.

All individuals displayed three to four host protective haplotypes and a high genetic score (Table 3)[248,263]. Those included protective human leukocyte antigen as (HLA)-B*57:01, HLA-B*58:01, HLA-B*27:05 or HLA-B*14:02[248,263], as well as the T>C mutation in the HLA-C polymorphism (rs9264942) in homozygosis, associated with lower plasma viremia and increased levels of HLA-C expression[263].

3a.3.2. Viral persistence

To evaluate the extent of viral persistence in the EEC, we used the most sensitive assays available and compared the values obtained with samples from individuals on cART[264]. The quantification of total HIV-1 DNA in infected $CD4^+$ T cells, which are believed to constitute most of the latent reservoir in peripheral blood, showed detectable, albeit extremely low (median of 10.4 and interquartile range [IQR; 9.1–23.0] copies/10^6 $CD4^+$ T cells) total HIV-1 DNA in all individuals (Fig. 22a). Similar numbers of provirus-containing cells were detected in frozen samples obtained 11 to 13 years earlier (median of 12.5 [2.5–31.3] copies/10^6 $CD4^+$ T cells) suggesting the stability of the viral reservoir for long periods of time (Fig. 23).
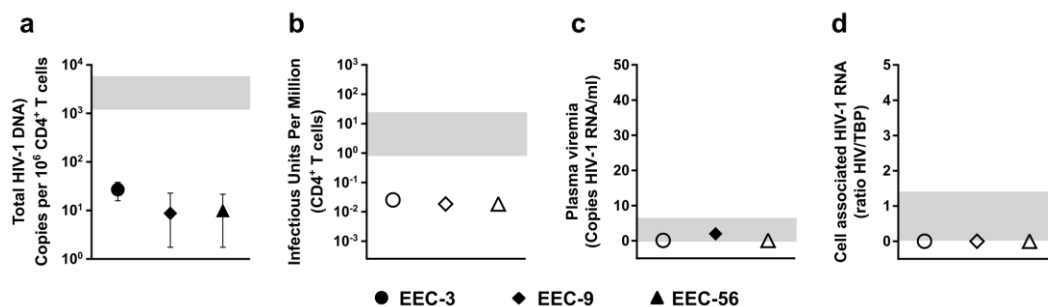
Figure 22. HIV-1 reservoir quantification. (**a**) Total HIV-1 DNA, (**b**) Infectious Units per million cells (IUPM) in a qVOA assay, (**c**) ultrasensitive plasma viral load and (**d**) cell associated HIV-1 RNA (caHIV-1 RNA). Open symbols indicate undetectable values. Light grey bands are the interquartile range from standard HIV-1-infected individuals under treatment[264].
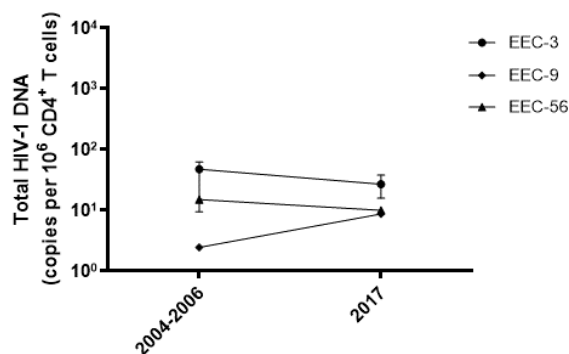
Figure 23. Total HIV-1 DNA evolution. Values represented are from the sample from 2017 and a sample 11-13 years before (2004-2006).

As total HIV-1 DNA indistinctly measures both defective and replication-competent forms, we quantified the frequency of IUPM in CD4$^+$ T cells in a co-cultured qVOA. We did not detect any replication competent virus in a large amount (28–63 million cells) of highly activated CD4$^+$ T cells from the individuals, indicating values of IUPM below 0.025 (Fig. 22b).

The ultrasensitive viral load assay (usVL) only detected 2 copies of HIV-1 RNA per ml of plasma in EEC-9 and in the other two individuals it was undetectable in 9 ml of plasma (Fig. 22c). Quantification of cell-associated HIV-1 RNA (caHIV-1 RNA) showed absence of viral transcription in peripheral CD4$^+$ T cells in the three individuals in more than three million tested cells (Fig. 22d).

3a.3.3. Sequence analysis, genetic variability, and evolutionary dynamics of viral populations

HIV-1 viral replication is inevitably linked with viral diversity and evolution[265]. Therefore, we retrospectively explored genetic evolution in HIV-1 DNA samples from the EEC from the last 15 years. Multiple *env* sequences were obtained from different time points in EEC-3 and EEC-56, but only two sequences in EEC-9 from a single time point were obtained. Of all the recovered sequences, only the two sequences from EEC-3 were hypermutated, compared to greater numbers found in other HIV-1-infected subjects or individuals on cART[266,267] which support the very low viral replication level in these EEC. In addition, we intended to obtain near full-length genome sequences from proviral DNA. Only one complete genome was recovered in EEC-3 (GeneBank MN055643). The remaining sequences (92%) showed important deletions throughout the genome mostly in *pol* to *env* genes. These data supported the notion that most provirus in these individuals were defective.

The phylogenetic trees showed very short branches with the major presence of viral sequences with no distance to the most common recent ancestor (Fig. 24a). This pattern was already evident in the first sample and it was maintained in different samples over the years. There were isolated small clusters of sequences showing longer branches in EEC-3 and EEC-56, which were not hypermutated. However, these viral populations were evolutionary dead ends and did not appear in further samples.

Moreover, the genetic variability of the viral quasispecies showed a very restricted genetic diversity, estimated to be around 0.010 ± 0.003 substitutions per nucleotide (s/n) throughout the entire follow-up (Fig. 24b). The degree of *env*-based heterogeneity was up to eight times lower in EEC than in other HIV-1-infected groups with controlled viral replication, including individuals on cART, viremic LTNP or transient EC one year before the loss of control (FDR<0.05) (Fig. 24b). Altogether, the practically null viral genetic evolution and extremely low complexity of the viral populations support the absence of viral replication in our EEC group over 25 years.
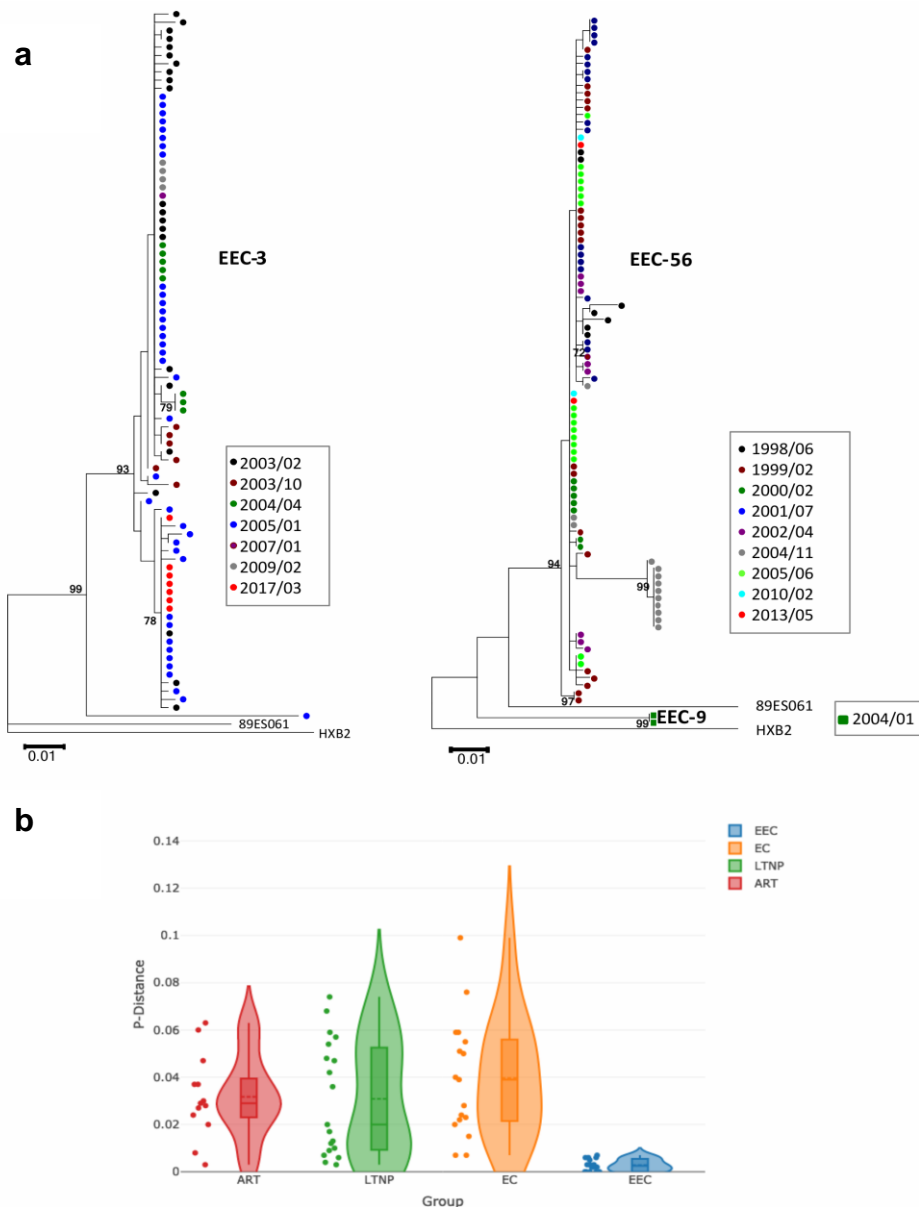
Figure 24. Genetic variability and evolutionary dynamics of viral populations. (**a**) Phylogenetic trees with *env* gene sequences of the individuals during follow-up. The evolutionary history was inferred in trees by using the Maximum Likelihood method based on the General Time Reversible model. The percentage of trees in which the associated taxa clustered together with values over 70% is shown next to the branches. Initial tree for the heuristic search was obtained by applying the

Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6. Different colors were used to indicate the sampling time. (**b**) Genetic variability analysis of samples from different groups of HIV-1-infected individuals with a controlled infection.

3a.3.4. Evolution of immune responses

3a.3.4.1. HIV-1 specific T-cell responses and poly-functionality

The magnitude of Gag-specific T-cell response was calculated as the percentage of Gag-specific CD8^{+} T-cells producing at least one intracellular cytokine (Tumor necrosis factor (TNF), IL-2 and/or IFNγ). EEC presented higher levels of Gag-specific total CD8^{+} T-cells when compared with HIV-1-infected individuals on suppressive cART (Fig. 25a). Simultaneous release of the three intracellular cytokines was determined using the poly-functionality index (pINDEX). The pINDEX in Gag-specific total CD8^{+} T-cells in EEC was higher (p=0.017) when compared with individuals on suppressive cART (Fig. 25b).
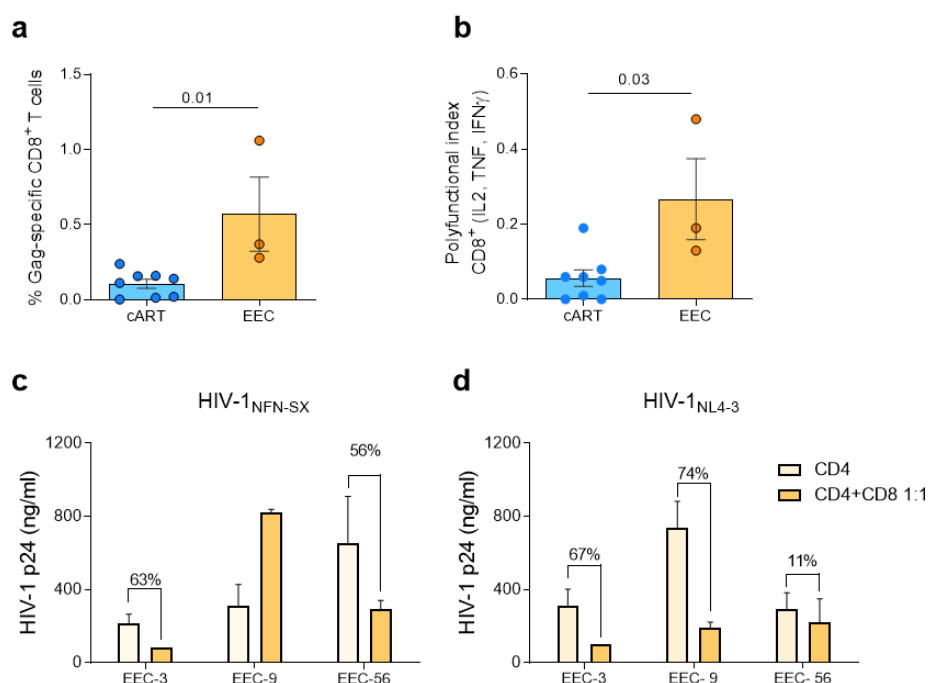
Figure 25. Cellular Immune responses. (**a**) Total CD8^{+} T-cell Gag-specific response from EEC and HIV-1-infected individuals on suppressive cART. (**b**) INDEX of polyfunctionality (pINDEX) of Gag-specific total CD8^{+} T cells from EEC and HIV-1-infected individuals on suppressive cART based on the proportions of cells producing intracellular combinations of IFN-γ, TNF, and IL-2. (**c,d**) Viral inhibition assay. Assay of the *ex vivo* ability of CD8^{+} T cells to inhibit superinfected autologous CD4^{+} T cells of the three individuals. The figure shows day 7 of an infection with a laboratory viral strain (**c**) HIV-1$_{NFN-SX}$ (CRR5-tropic) and (**d**) HIV-1$_{NL4-3}$ (CXCR4-tropic). Percentage of inhibition of CD4^{+} T cells vs CD4^{+}:CD8^{+} T cells is indicated in each individual. (**a,b**) Differences between groups were determined by Mann-Whitney U test.

3a.3.4.2. CD4⁺ susceptibility and CD8⁺ viral inhibition

To analyze the susceptibility of the target cells to HIV-1 infection, we pulsed autologous CD4$^+$ T cells from each individual with laboratory-adapted R5- and X4-tropic viral strains for 7 days (Fig. 25 c,d). This assay shows that CD4$^+$ T cells from the individuals were susceptible to HIV-1 infection.

When we added autologous CD8$^+$ T cells to these co-cultures, we observed a reduction of viral replication from 11 to 74% with the X4-tropic virus and up to 63% with the R5 tropic virus (Fig. 25 c,d). Thus, host CD4$^+$ T cells are not intrinsically refractory to HIV-1 infection with R5 or X4-tropic viruses, and host CD8$^+$ T cells are effective in suppressing viral replication *ex vivo*.

3a.3.4.3. Inflammation biomarkers

EEC had lower levels of hsCRP, β2-microglobulin and D-dimer levels than individuals on cART, but comparable to HD (Fig. 26a–e). IL-6 and sCD163 were similar between EEC and cART groups, despite the levels of IL-6 and sCD163 were higher than in HD, probably due to previous history of HCV and residual expression of HIV-1 antigens.
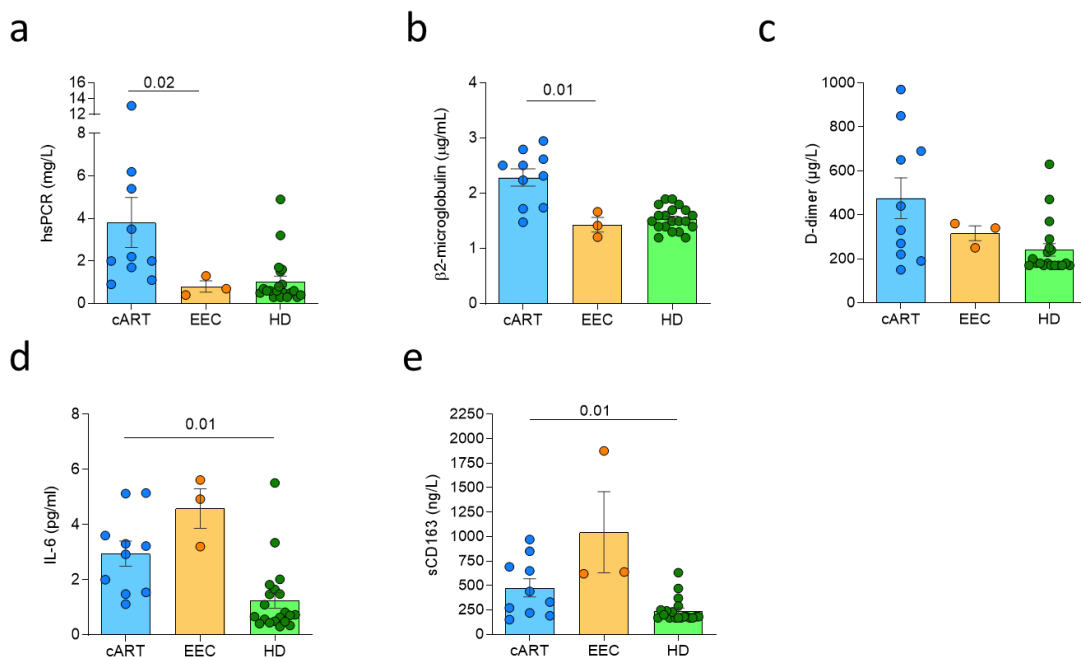
Figure 26. Inflammation biomarkers. Different inflammatory markers were analyzed including (**a**) hsCPR, (**b**) β2-microglobulin, (**c**) D-dimer, (**d**) IL-6 and (**e**) sCD163. EEC individuals were compared with HIV-1-infected individuals on cART and non-HIV-1-infected healthy donors (HD).

3a.3.4.4. HIV-1 specific antibody levels

All EEC included in this study had detectable HIV-1 specific antibodies measured by a detuned ELISA with no differences in comparison with individuals on cART (Fig. 27a). Similarly, the quality of their antibodies measured by the Lag Avidity assay showed no differences with individuals on cART (Fig. 27b)
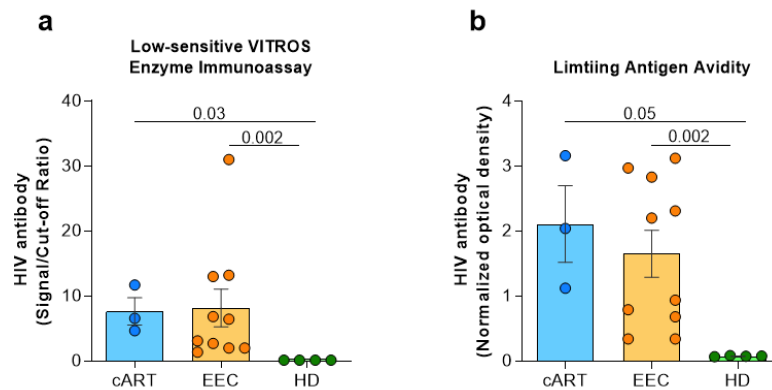
Figure 27. Measurement of HIV-1 specific antibodies. (**a**) Levels of HIV-1 specific antibodies measured by a detuned low-sensitivity and (**b**) and limiting antigen avidity assay. EEC individuals were compared with HIV-1-infected individuals on cART and non-HIV-1-infected healthy donors (HD).

3a.4. DISCUSSION

Herein we characterize three EEC who spontaneously controlled HIV-1 replication and its associated clinical pathogenesis for more than 25 years. According to our observations, the almost total absence of viral diversity and evolution analyzed during at least 15 years, probably due to a replication-impaired viral reservoir, adds to previously described protective host genetic factors and effective immune responses.

All three EEC individuals included in this study showed a consistently low viral DNA reservoir in peripheral CD4$^+$ T cells that was 50 times lower than in individuals on cART using the most sensitive techniques[264]. Total HIV-1 DNA, caHIV-1 RNA, residual plasma viremia, and IUPM were also lower than other values independently reported for EC[268,269]. Moreover, HIV-1 *env* sequences had nearly null viral genetic evolution, extremely low complex populations supporting the absence of viral replication over 25 years.

This replication impairment is further supported by the variation in only two nucleotides between the only complete genomic sequence of EEC-3 and another one obtained 12 years earlier[256]. The lack of sequence heterogeneity despite a long-term untreated infection might reflect a combination of a slow-evolving virus and the clonal expansion of infected immune cells[111] as suggested by the presence of identical viral clusters in the phylogenetic trees. Finally, the low levels of proviral DNA seemed to be largely defective (>92%), since they had no capacity to generate viral mRNA, viral particles or replication competent virions, implying limited viral persistence in these EECs. The lack of viral replication, as shown by different markers and low hypermutation, despite detectable HIV-1 DNA has been mainly associated with the presence of proviral defective genomes and the stability of the viral reservoir[132,160,256].

Low population size and viral diversity are associated with low replication and low viral fitness[270]. These viral characteristics favors important fitness losses, because of the irreversible accumulation of deleterious mutations, known as the Muller´s ratchet effect as observed *in vitro*[271]. The combination of these factors is able to prevent viral fitness recovery, and even if some remaining virus are present

in the body, it will not generate replication competent viruses because viral populations do not have enough variability to recover fitness[272]. The low diversity and population size could have contributed to the maintenance of the control of viral replication in the participants during the follow-up. All study individuals showed the lowest viral diversity values among comparative groups of individuals on cART or LTNP with viral load control. In addition, a previous study showed that low diversity and lack of viral evolution was highly correlated with permanent viral control in persistent EC[249]. On the contrary, transient EC who lost control during the follow-up showed higher levels of genetic diversity[249]. Thus, extremely low viral diversity could be a convenient prognostic marker for the identification of these EEC.

The functional characterization of viruses from EEC has been limited by the impossibility of complete virus recovery. In a previous study, EEC-56 showed the major presence of 228 nucleotide deletions in the 5′ LTR–*gag* region; in EEC-3 a 247 nucleotide deletion was positioned in *pol* gene up to the *vif* orf[256]. These deleted genomes became dominant during follow-up[256]. In other studies, cloned *env* sequences from EEC-3 in recombinant viruses showed that displayed a very limited and retarded replication capacity[273,274], similarly to what has been observed in *env* recombinant viruses from some EC[275].

According to the data reported in this work, viral antigens and/or truncated viral proteins could be generated in these individuals from defective genomes or from new alternative spliced HIV-1 RNA variants with translationally competent ORFs as reported in HIV-1-infected individuals on cART[143]. The apparent limited amount of peripheral viral antigen might be associated with the detectable levels of HIV-1 specific antibodies. Likewise, both frequency and poly-functionality of Gag-specific CD8^{+} T-cell responses were higher in EEC when compared with HIV-1-infected individuals on cART and similar or above the median of a group of persistent EC (with more than 18 years of controlled infection), and higher than those of transient EC[249]. The CTL responses supported the capacity of the immune system to mount effective adaptive immune responses as confirmed by the *ex vivo* viral inhibition assay.

Inhibition from autologous CD8^{+} T cells was similar to previous described EC and higher than comparisons with non-HIV infected donors[276]. Innate immune responses seem to be relatively normalized as for the quantification of plasma inflammation biomarkers when compared to HD individuals. Only IL-6 and sCD163 were slightly superior if compared HD to HIV-1-infected individuals on cART. The obtained values were in the range of those found in independent cohorts of EC[277,278]. This could be due to the previous long-term story of HCV infection, and in fact, sCD163 has been proposed as a marker of liver fibrosis[279]. This is a substantial difference with previously published EC profiles where despite the viral control, inflammation levels were maintained over time[274,280]. Whether this could be a distinctive marker easy to assay for EEC needs to be examined in extended populations, but previous works with persistent EC suggested this idea[249,281]. The interplay of T cells with the preserved number and functions of innate cells might have been a critical factor for the maintenance of the high HIV-1 specific T-cell responses in these individuals[282]. Although there is slight decline in CD4^{+} T-cell levels in EEC-9, this decrease can be considered physiological with aging, during the 20 years of follow-up and with the fact that the studied individuals are now over 60 years old, and moreover this diminution is comparable with European uninfected individuals[283].

We can only speculate how these HIV-1-infected individuals have resulted in this EEC phenotype of potential functional viral suppression. Thus, primary infection might have occurred with a low fitness viral founder strain, or alternatively, initial innate immune responses might have shaped the selection of an unfit virus. Host genetic factors, including those related with the HLA function, and cellular-adaptive immune responses might have further contributed to this clinically unusual non-progressive profile. Although these are naturally occurring cases, and not the result of a clinical intervention, they provide knowledge on how to achieve a functional cure for HIV-1 infection. Based on all these evidences we believe that these individuals do not require any cART. Perhaps other individuals, most probably within the persistent EC, LTNP-EC, and EEC groups with similar host genetics factors (at least three to four protective alleles), immune responses (with a pINDEX>0.50) and viral

factors (with stable viral diversity (below 0.010 ± 0.003 (s/n) throughout the entire follow-up) could also have spontaneously achieved a HIV-1 functional cure[284].

Four previous cases of durable control over HIV-1 replication without cART have been reported[71]. While those cases also had extraordinarily low HIV-1 burdens, they differed from ours in their shorter follow-up (median of 9 years since HIV-1 diagnosis) and their weak reactive Western blots. A more recent study in HIV-1 Controllers (HIC) followed-up for a median of 21 years has shown a similar small HIV-1 blood reservoir in the presence of weak T-cell activation levels[250]. Our study adds three new additional characteristics to this EEC phenotype: (i) even more time since HIV-1 diagnosis without cART, (ii) low viral diversity and lack of viral evolution over the years, and (iii) inflammatory markers in peripheral blood similar to those in healthy donors.

In conclusion, we have defined a new subset of HIV-1-infected individuals termed as Exceptional Elite Controller who are able to control HIV-1 replication for longer than 25 years. The incidental accumulation of protective host factors, unfit and/or defective viruses that precluded viral evolution and diversification, low non-inducible reservoir, and effective adaptive immune responses, might have taken place simultaneously to achieve a spontaneous HIV-1 functional cure. This model of control suggests that new curative strategies would be successful combining approaches that achieve a reduction of the HIV-1 latent reservoirs, maintain extremely low viral diversity, an impaired viral fitness, and enhanced HIV-1 specific immune responses.

Chapter 3b. Extremely low viral reservoir in chronically treated HIV-1-infected individuals

This chapter corresponds to the manuscript:

__Gálvez C__; Urrea V; Dalmau J; Jimenez M; Clotet B; Monceaux V; Huot N; Leal L; González-Soler V; González-Cao M; Müller-Trutwin M; Sáez-Cirión A; García F; Blanco J; Martinez-Picado J; Salgado M. <u>Extremely low viral reservoir in treated chronically HIV-1-infected individuals</u>. EbioMed.2020;57:102830

3b.0. PRESENTATION

The study of the three EEC in chapter 3a showed that very low HIV-1 reservoir seems to be associated to low evolution and enhanced immune responses. However, it has been challenging to mimic this kind of functional cure for the great majority of HIV-1-infected individuals. Alternative factors associated with a reduced HIV-1 reservoir needs to be discovered. Therefore, a screening of HIV-1 DNA was done in a large cohort of HIV-1-infected individuals under cART with the aim of finding individuals with a very low reservoir like EEC, and stablish the Low Viral Reservoir Treated cohort (LoViReT). With LoViReT individuals, new factors associated with a reduced HIV-1 reservoir will be investigated.
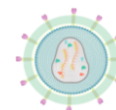
3b.1. INTRODUCTION

The main goal of the eradication strategies is reducing the latent viral reservoir to undetectable levels[285]. Therefore, factors related to the size, distribution, and stability of the viral reservoir are continuously being investigated. It has been postulated that the amount of HIV-1 DNA is a predictor of disease progression in primary infection[286] and during the natural course of HIV-1 infection[287].

Various studies suggest that early initiation of cART is an important factor in reducing the size of the viral reservoir[65,288], especially if initiated at Fiebig I stage[242]. Unfortunately, individuals are rarely treated during the acute phase, since most new diagnoses of HIV-1 infection are made at the chronic stage, when the reservoirs are more stable[289]. Eradication strategies need to be effective in the vast majority of treated chronically HIV-1-infected individuals. Several studies have described treated chronically infected individuals with low or even undetectable levels of total HIV-1 DNA[239,243,244]. However, no retrospective data have been reported on the joint proportion of individuals who achieve a low reservoir after initiation of treatment in both the acute and the chronic phases. Furthermore, the factors involved in achieving these low latency levels have not been investigated in depth.

In this study, we screened the total HIV-1 DNA reservoir in 451 treated HIV-1-infected individuals with suppressed plasma viremia for at least three years and stored cryopreserved PBMCs to establish the Low Viral Reservoir Treated cohort (LoViReT). We aimed to study the kinetics of these decreased reservoirs and to analyze associated clinical and immunological factors. To do so, we focused on a subset of LoViReT individuals who initiated treatment in the chronic phase of the infection (cp-LoViReT) in order to identify strategies that could be applied in the vast majority of treated HIV-1-infected individuals.

3b.2. METHODS

3b.2.1. Study participants

We retrospectively screened 451 HIV-1-infected individuals undergoing regular follow-up at Hospital Germans Trias i Pujol (n=319) and Hospital Clinic (n=132) in Barcelona. We included individuals under suppressive cART with undetectable viremia (HIV-1 RNA <50 copies/ml) for at least three years and with available cryopreserved PBMCs. Demographic and clinical data were collected from the clinical database. The characteristics of the study participants were similar to those of previously reported cohorts of individuals with low HIV-1 reservoirs in terms of sex, age, and time with infection[239,243,244]. Treated chronically infected participants were defined as individuals with more than six months between acquisition of HIV-1 and initiation of treatment.

All individuals provided their signed informed consent to participate in the study. The study was approved by the Ethics Committee at both recruiting hospitals (reference #: PI-014-083).

3b.2.2. Quantification of proviral HIV-1 DNA

The size of the proviral reservoir was measured in PBMCs for screening and in purified CD4+ peripheral T cells for the longitudinal analysis (Miltenyi Biotech) by ddPCR, as previously described in chapter 3A.

3b.2.3. Viral tropism

Viral co-receptor tropism was determined on proviral DNA by sequencing of HIV-1 GP120 hypervariable region 3 (V3). Tropism was predicted using the Geno2Pheno algorithm (GENAFOR, Bonn, Germany), with a false-positive rate (FPR) of 10% (non–R5-tropism: FPR ≤10%)[290].

3b.2.4. Immunophenotyping, activation and exhaustion markers in peripheral CD4+ and CD8+ T cells

For T-cell immunophenotyping, cells were incubated with Fixable Viability Stain 780 (APC H7), CD3 (SK7), CD4 (RPA-T4), and CD8 (SK1) monoclonal antibodies. T-cell maturation was based on the expression of CD45RA (HI100), and CD197/CCR7 (G043H7) was analyzed in cryopreserved PBMCs in order to define

T naïve (CD45RA$^+$CCR7$^+$; T$_N$), T central memory (CD45RA$^-$CCR7$^+$; T$_{CM}$), T effector cells (CD45RA$^-$CCR7$^-$; T$_{EF}$), and T Effector Memory RA$^+$ (CD45RA$^+$CCR7$^-$; T$_{EMRA}$). CD27 (O323) was used to differentiate effector cells into T effector memory (CD27$^-$ CD4$^+$ or CD27$^+$CD8$^+$; T$_{EM}$), and T terminally differentiated (CD27$^+$CD4$^+$ or CD27$^-$ CD8$^+$; T$_{TM}$). CD4 and CD8 T cells were also analyzed for expression of HLA-DR (L243) and CD38 (HIT2) to define activated cells (HLA-DR$^+$CD38$^+$); expression of CD279/PD-1 (EG12.2H7) was analyzed to measure exhausted cells (CD279$^+$). The gating strategy is shown in Fig. 28.

Samples were acquired in a BD LSR Fortessa and analyzed using FlowJo software (Tree Star). T cells were analyzed with automated detection of marker cutoffs based on fluorescence-minus-one controls (OurFlow platform using R packages).
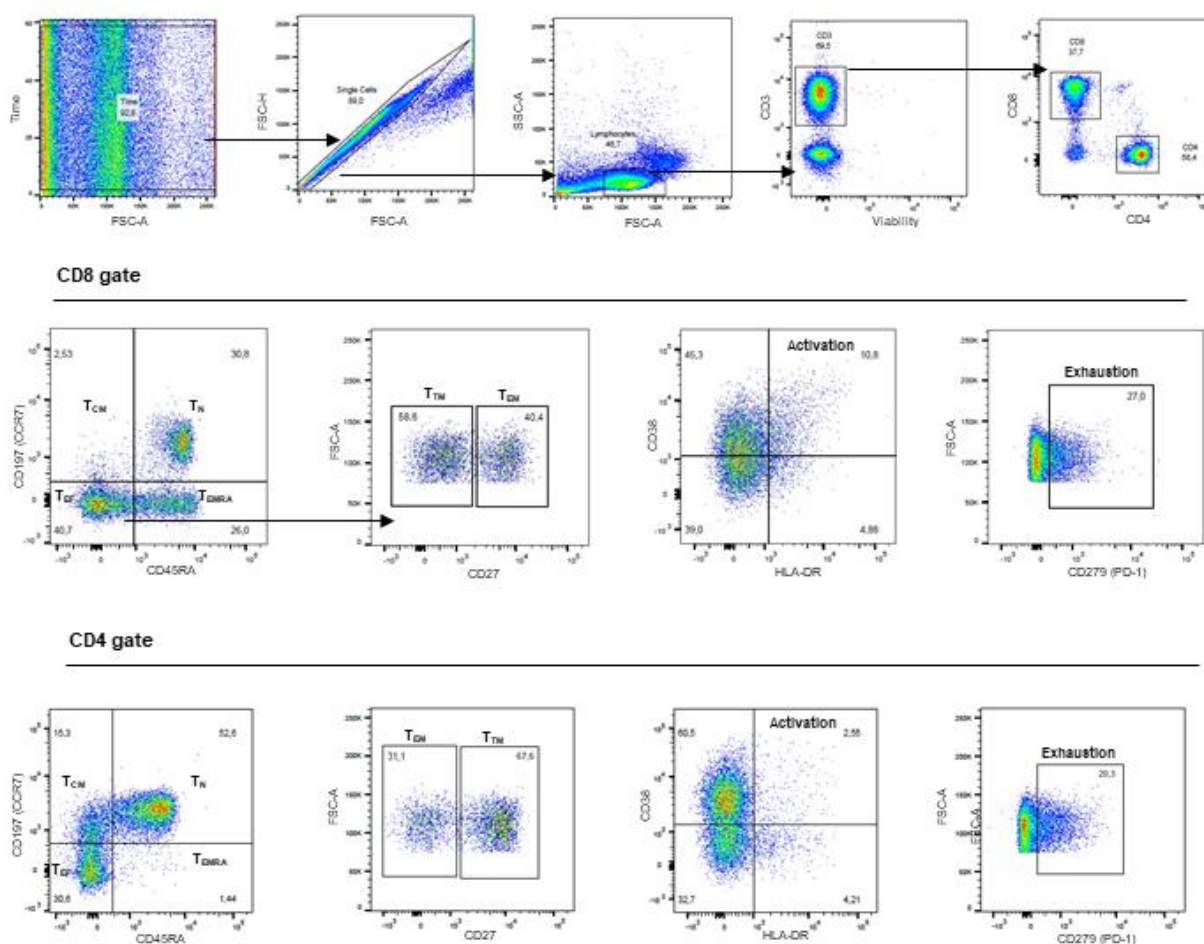
Figure 28. Gating strategy for T-cell analysis. PBMCs were acquired and gated according to (1) time to ensure homogeneous acquisition, (2) FSC-A and FSC-H to select singlets, (3) SSC-A and FSC-A to determine cell morphology, and (4) CD3 and viability staining to select living T cells. CD8

and CD4 cells were identified from this previous gate. For CD8 and CD4 T cells, maturation, activation, and exhaustion were defined as previously described.

3b.2.5. Viral inhibition by CD8⁺ T and NK cells

PBMCs were thawed and cultured overnight in RPMI 1640 containing GlutaMAX, 20% FCS, penicillin (10 IU/ml), and streptomycin (10 µg/ml). Analyses were performed as previously described[291]. Briefly, CD4⁺ T cells were purified by positive selection with antibody-coated magnetic beads (EasySep Human CD4 Positive Selection, StemCell Technologies). The elution containing CD4-depleted PBMCs was split and used for purification of CD8⁺ T-cells and NK cells by negative selection (EasySep Human CD8⁺ Cell Enrichment, EasySep Human NK Enrichment, StemCell Tecnhologies). Cells were separated using a RoboSep instrument (Stemcell Technologies).

CD4⁺ T cells were activated for 3 days in the presence of 4 µg/ml PHA-L (Roche) and 100 IU/ml IL-2 (Human IL-2 IS, premium grade, Miltenyi Biotech). During this period, NK cells were cultured in the presence of IL-15 at 0.1 ng/ml and CD8⁺ T cells in the absence of cytokines. After 3 days of culture, living activated CD4⁺ T cells were seeded in a 96-U-well plate (10^6 cells/ml in triplicate) alone or in the presence of CD8⁺ T cells or NK cells (1:1 ratio) and then exposed to HIV-1$_{BaL}$ (CCR5 tropic strain) (10 ng p24/ml). After spinoculation (1200 x g for 1 h at room temperature), cells were cultured for 1 h at 37ºC and washed before culture in the presence of IL-2 (100 IU/ml). Culture supernatants were removed, and the media culture was replenished at day 3. Levels of p24 in culture supernatants were analyzed at day 3 after infection for co-culture with NK cells and at day 7 after infection for co-cultures with CD8⁺ T-cells using an ELISA p24 assay (HIV-1 p24 ELISA kit, XpressBio).

3b.2.6. Quantification of inflammation biomarkers

Concentrations of the pro-inflammatory or homeostatic cytokines IL-2, IL-6, IL-7, IL-10, IL-27, and Interferon gamma-induced protein 10 (IP10), as well as the coagulation biomarker D-dimer, were quantified in plasma using a bead-based multiplex immunoassay (ProcartaPlex, eBioscience) according to the manufacturer's recommendations. Measurements were performed using a Luminex

200 instrument (Luminex Corp.) and analyzed using a standard curve for each cytokine.

3b.2.7. HIV-1 antibodies quantification

Specific HIV-1 antibodies were measured in plasma samples with a low sensitive (LS) version of the VITROS anti-HIV-1 assay, and a limiting antigen avidity assay (Lag-Avidity) (Sedia), as previously described in Chapter 3a.

3b.2.8. Statistical analysis

The clinical characteristics of the study population were presented as percentages for categorical variables and as median and IQR for continuous variables. The association between clinical parameters with low levels of cell-associated total HIV-1 DNA were analyzed using univariate and multivariate logistic regression models; the odds ratio, p-value, and C-index are reported. Variables associated with the LoViReT status (p<0.1) were considered candidates for the final model. The multivariate model was built using a stepwise procedure and by selecting the variables associated with LoViReT. The parameters assessed include gender, age at diagnosis, mode of infection, AIDS events, maximum viral load reported, CD4 nadir, time since HIV-1 diagnosis, viral blips (<500 copies/ml), detectable plasma viral load (pVL; any value above LOD), time with detectable pVL, tropism, and the accumulated pVL score calculated as the area of the positive pVL plot over time and normalized (divided) by the follow-up time. The model was validated internally using a 10-fold cross-validation, and predictive ability was assessed using the C-index (equivalent to the AUC under the ROC curve).

In the longitudinal analysis, clinical differences between groups were assessed using the Mann-Whitney test in the case of continuous variables and the Fisher exact test in the case of categorical variables. Clinical variables were matched between LoViReT individuals and controls using the random forest algorithm in order to avoid confounding factors associated with low HIV-1 DNA levels. Random forest[292] is a popular machine learning algorithm based on ensembles of Classification and Regression Trees (CART) algorithm (regression and classification trees), which provide good predictive performance and low overfitting. In addition to the prediction model, the algorithm provides a proximity measure

matrix between observations that can be used in matching[293]. In order to produce balanced treatment and control groups in terms of all relevant clinical factors, we selected a set of controls based on the RF proximity measure. Differences within groups were assessed using the Wilcoxon signed-rank test.

The analyses were performed with R (v3.4) and GraphPad (v5.01).
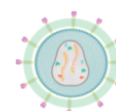
3b.3. RESULTS

3b.3.1. Characteristics of the screening population

We analyzed samples from 451 individuals with more than three years under suppressive cART. Their characteristics are summarized in Table 4.

Table 4. Clinical characteristics of the individuals included in the study.

Characteristic	N (%)	Median [IQR]
Male sex	377 (83.6)	
Region of origin		
Spain	236 (81)	
Europe	17 (6)	
America	34 (12)	
Africa	4 (1)	
Age at diagnosis		31 [27-37]
Mode of infection		
MSM	252 (58)	
Heterosexual	87 (20)	
Intravenous drug use	55 (13)	
Other	40 (9)	
AIDS events		
Yes	46 (10)	
No	405 (90)	
Zenith viral load ($\log_{10}$ copies/ml plasma)		4.9 [4.3-5.3]
CD4 nadir (cells/µl)		273 [159-358]
Time since HIV-1 diagnosis (years)		13 [7-19]
Viral blips		0 [0-1]
Virological failures		0 [0-2]
Tropism		
R5-tropic	329 (73)	
Non-R5 or dual	93 (21)	
Viral subtype		
B	403 (96)	
Non-B	19(4)	
Total HIV-1 DNA (copies/10^6 PBMCs)		158.5 [78.7-313.4]
At the time of HIV-1 DNA measurements		
Age		46 [41-51]
Time suppressed (years)		5.6 [4-8]
CD4 T cells (cells/µl)		676 [495-891]
CD8 T cells (cells/µl)		766 [567-1022]
CD4/CD8 ratio		0.9 [0.6-1.2]

$\log_{10}$ proviral HIV-1 DNA in PBMCs was normally distributed (Fig. 29a). Median HIV-1 DNA was 158.5 copies/10^6 PBMCs [IQR, 79-313]. A total of 42 individuals were under the 10th percentile (<50 HIV-1 DNA copies/10^6 PBMC) (9.3%), including four (0.9%) who had levels below the LOD for the two sets of primers used. They were all included in the "LoViReT" (Low Viral Reservoir Treated) cohort. The distribution of total HIV-1 DNA and proportion of LoViReT individuals were similar between the two recruiting centers (Fig. 29b).
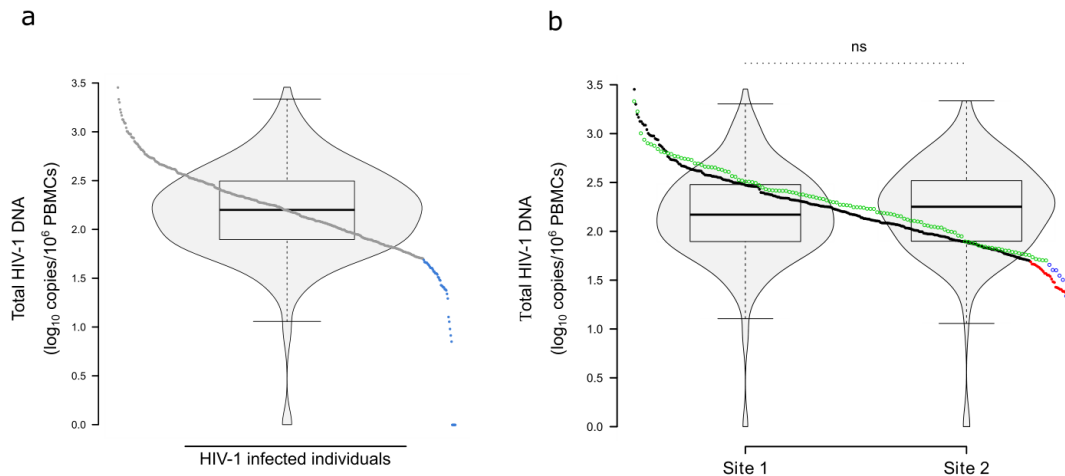
Figure 29. Total HIV-1 DNA. (**a**) Total HIV-1 DNA of 451 individuals after screening with ddPCR. Individuals with <50 copies/10^6 PBMCs are shown in light blue. (**b**) Comparative distribution of total HIV-1 DNA between the 2 recruiting centers LoViReT individuals are shown in red for site 1, and in blue for site 2.

The clinical histories of LoViReT individuals confirmed that 28 individuals were treated in the chronic phase (>six months since HIV-1 acquisition) and were defined as chronic phase LoViReT individuals (cp-LoViReT) (Fig. 30). Out of the remaining LoViReT individuals, six were treated in the acute stage, and eight lacked proven information of their time with infection at initiation of cART.
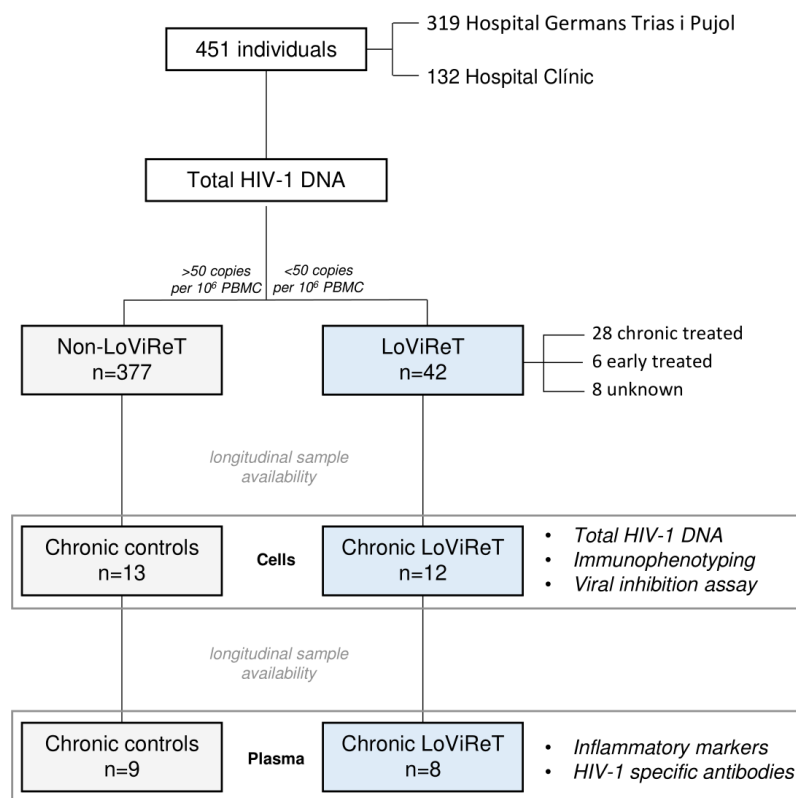
Figure 30. Study design flow-chart.
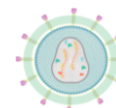
3b.3.2. Factors related to the LoViReT status

Clinical, immunological, and virological data were collected to search for factors predicting whether the subject would belong to the LoViReT cohort (Table 5). In the univariate analysis, being in the LoViReT cohort was associated with different parameters related to a lower pVL, and higher CD4^{+} T cells (Table 5). In the multivariate regression model, only a lower maximal reported pVL, fewer detectable pVLs during cART and higher CD4^{+} nadir, remained significantly associated with LoViReT status (Table 5). Using the c-index method to evaluate the predictive capacity of the multivariate model, we observed a goodness of fit of 0.72 after cross-validation. This is considered a moderate predictive capacity.

Thus, LoViReT individuals seemed to have lower peaks of pVL with a higher CD4^{+} T-cell nadir in the pre cART period followed by fewer detectable pVLs during their clinical follow-up. However, this multivariate model had limited predictive capacity.

Table 5. Clinical associations with low levels of total HIV-1 DNA (LoViReT status).

Clinical variables	Univariate		Multivariate	
	OR [95% CI]	p-value[*]	OR [95% CI]	p-value[*]
Gender (Male)		0.981		
Age at diagnosis		0.642		
Mode of infection		0.782		
AIDS events		0.749		
Maximum viral load reported $\log_{10}$	0.67 [0.51-0.89]	**0.007**	0.66 [0.49-0.89]	**0.0069**
CD4 nadir (multiples of 100)	1.47 [1.20-1.81]	**<0.001**	1.46 [1.19-1.81]	**0.0003**
Time since HIV-1 diagnosis		0.177		
Viral blips (<500 copies/ml)	0.64 [0.34-1.05]	0.083		
Detectable pVL (>50 copies/ml)	0.72 [0.55-0.90]	**0.002**	0.79 [0.60-0.98]	**0.0286**
Time with detectable pVL (years)	0.76 [0.58-0.94]	**0.008**		
Tropism		0.394		
Accumulated viral load score[&]	0.78 [0.62-0.95]	**0.013**		
At the time of HIV-1 DNA measurements				
Age		0.531		
Time suppressed (years)		0.902		
CD4 T cells (multiples of 100)		0.648		
CD8 T cells (multiples of 100)		0.246		
% CD4 T cells		0.344		
% CD8 T cells		0.262		
CD4/CD8 ratio		0.217		

[*]p-values based on likelihood ratio test; [&] Calculated as area under de curve

3b.3.3. Longitudinal analysis of HIV-1 DNA reservoirs

For further analyses, we focused on the cp-LoViReT population. Thus, we aimed to unravel whether the cp-LoViReT reservoir decreased as a consequence of cART or whether these individuals had lower reservoirs before initiation of cART.

According to sample availability, we selected a subgroup of 12 cp-LoViReT and compared them with 13 treated chronically infected matched controls (>50 HIV-1 DNA copies/10^6 PBMCs) to perform a retrospective longitudinal reservoir analysis in purified CD4$^+$ T cells. We previously ensured that there were no differences in clinical parameters between cp-LoViReT and controls (Table 6).

Table 6. Clinical characteristics of the individuals included in the longitudinal study.

	LoViReT group (n=12)	Control group (n=13)	p-value
Age at diagnosis (years), median [IQR][a]	33 [28-35]	33 [31-37]	0.35
Females, n (%)[b]	1 (8.3)	0 (0)	0.48
Mode of infection MSM, n (%)[b]	10 (83.3)	13 (100)	0.22
Time since diagnosis (years), median [IQR][a]	5.6 [5.1-7.6]	6.6 [5.5-7.0]	0.46
Time of viral load suppression (years), median [IQR][a]	4.8 [3.9-6.8]	4.9 [3.9-5.7]	0.77
Viral load			
Zenith (log copies/ml plasma), median [IQR][a]	5.0 [4.8-5.3]	4.9 [4.6-5.3]	0.76
Viral load sample pre cART ($\log_{10}$ copies/ml plasma), median [IQR][a]	4.1 [4.9-5.2]	4.9 [4.1-5.2]	0.99
CD4 T cell count			
Nadir (cells/µl), median [IQR][a]	422 [330-488]	409 [351-612]	0.81
Absolut (cells/µl) pre-cART, median [IQR][a]	622 [384-774]	477 [385-630]	0.25
Absolut (cells/µl) 5 years on cART, median [IQR][a]	802 [703-938]	808 [660-1069]	0.94
Antiretroviral treatment			
Number of different regimens, median [IQR][a]	3 [1-5]	2 [1.5-3]	0.34
Number of different families, median [IQR][a]	2.5 [2-3]	2 [2-3]	0.57
Nucleoside reverse transcriptase inhibitors, n (%)[b]	12 (100)	13 (100)	0.99
Non-nucleoside reverse transcriptase inhibitors, n (%)[b]	9 (75)	10 (77)	0.99
Protease inhibitors, n (%)[b]	6 (50)	6 (46)	0.99
Integrase inhibitors, n (%)[b]	4 (33)	2 (15)	0.38
Entry inhibitors, n (%)[b]	2 (17)	1 (8)	0.6

[a]p-value between groups: Mann-Withney. [b]p-value between groups: Fisher Exact Test.

Total HIV-1 DNA was measured before cART and in several samples after initiation of cART (median: 4 [IQR: 4-5] samples per individual. Fig. 31a shows the dynamics of total HIV-1 DNA in all the samples analyzed. All participants had detectable HIV-1 DNA before therapy, thus discarding technical primer-mismatch hybridization issues. We observed a phase of faster decay during the first 18 months on cART, followed by a slower decay. When we analyzed the time points separately, we observed that before initiation of cART, cp-LoViReT already harbored significantly lower levels of HIV-1 DNA (p=0.002) (Fig. 31b). The differences observed before cART were independent from pVL, since no statistically significant differences were observed between the groups (Table 6).

After 18 months of treatment, a pronounced decline in HIV-1 DNA was observed in all those studied, although median values were lower in cp-LoViReT than in controls (p<0.001). Finally, after five years on cART, samples from both groups
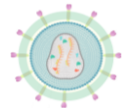
showed the greatest differences within the study (p<0.001). cp-LoViReT reached a median of 54 HIV-1 DNA copies/10^6 CD4$^+$ T cells, which is comparable to the values we observed in the initial PBMC screening.

When we compared the decay in the HIV-1 reservoir before and after five years on cART, we observed a significantly greater decrease in cp-LoViReT individuals than in controls (16- vs. 5-fold respectively, p<0.001) (Fig. 31c). In addition, a significant positive correlation was observed between these two time points (rho=0.76, p<0.001) (Fig. 31d).

Altogether, we observed that cp-LoViReT individuals had low viral reservoirs before initiation of cART, as well as enhanced decay during treatment.
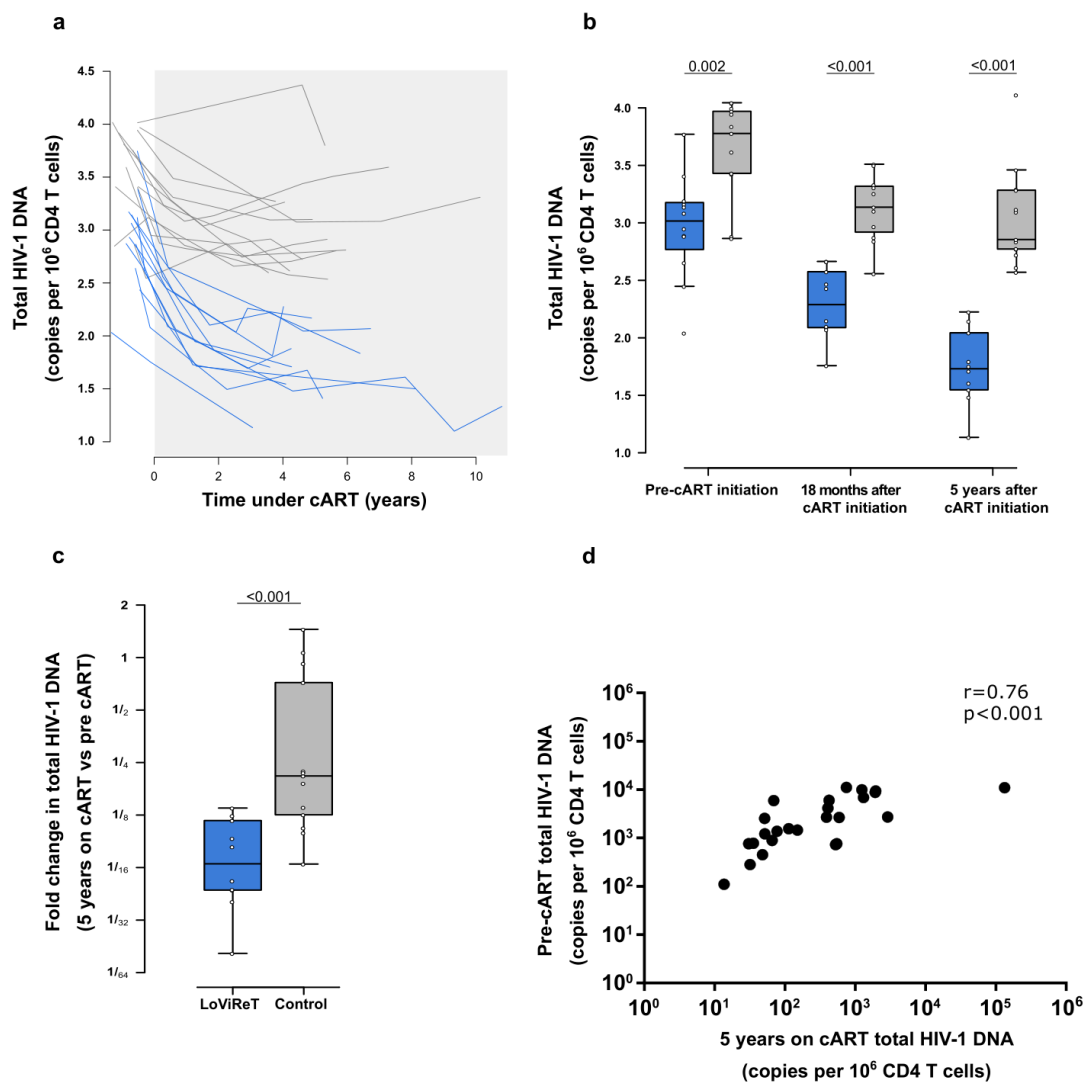
Figure 31. Longitudinal measure of total HIV-1 DNA in CD4$^+$ T cells by ddPCR. (**a**) Decay in total HIV-1 DNA before and after initiation of cART. The light grey box indicates the period under cART. (**b**) Box plot of total HIV-1 DNA at three different time points: pre-cART, 18 months after initiation of cART, and 5 years after initiation of cART. (**c**) Fold change decay in the sample before initiation of

treatment and after 5 years of cART. (**d**) Spearman correlation for total HIV-1 DNA pre-cART and after 5 years of cART.

3b.3.4. Characterization of T-cell subsets, activation, and exhaustion

Immunophenotyping of T cells was performed in the samples used for the longitudinal reservoir analysis. As expected, we found a significant increase in the frequency of CD4$^+$ T cells and a significant decrease in the frequency of CD8$^+$ T cells after initiation of cART, thus leading to a significant recovery of the CD4/CD8 T-cell ratio.

Analysis of maturation subsets in CD8$^+$ T cells revealed the expected significant increase in the frequency of T$_N$ cells after initiation of therapy in both groups, with similar rates of increase; however, the frequency was higher at month 18 in cp-LoViReT than in controls (p=0.043) (Fig. 32a-b). Although we did not observe significant differences between the groups in the frequency of T$_{CM}$ (Fig. 32c), we did detect a lower frequency of T$_{TM}$ and T$_{EMRA}$ cells in before initiation of cART in cp-LoViReT than in controls (p=0.011 for T$_{TM}$ and p=0.016 for T$_{EMRA}$). This association disappeared when cART was introduced (Fig. 32d-f).

Activated (CD38$^+$HLA-DR$^+$) and PD-1$^+$ CD8$^+$ T cells decreased significantly over time in both groups (Fig. 32g-h). However, no significant differences between groups were observed.

In CD4$^+$ T cells, we did not observe any significant difference between cp-LoViReT and controls over time in any T-cell maturation subset (Fig. 33a-f). Activation, defined as co-expression of CD38$^+$HLA-DR$^+$, and PD-1 expression levels decreased overtime in both groups with no intergroup differences (Fig. 33g-h).
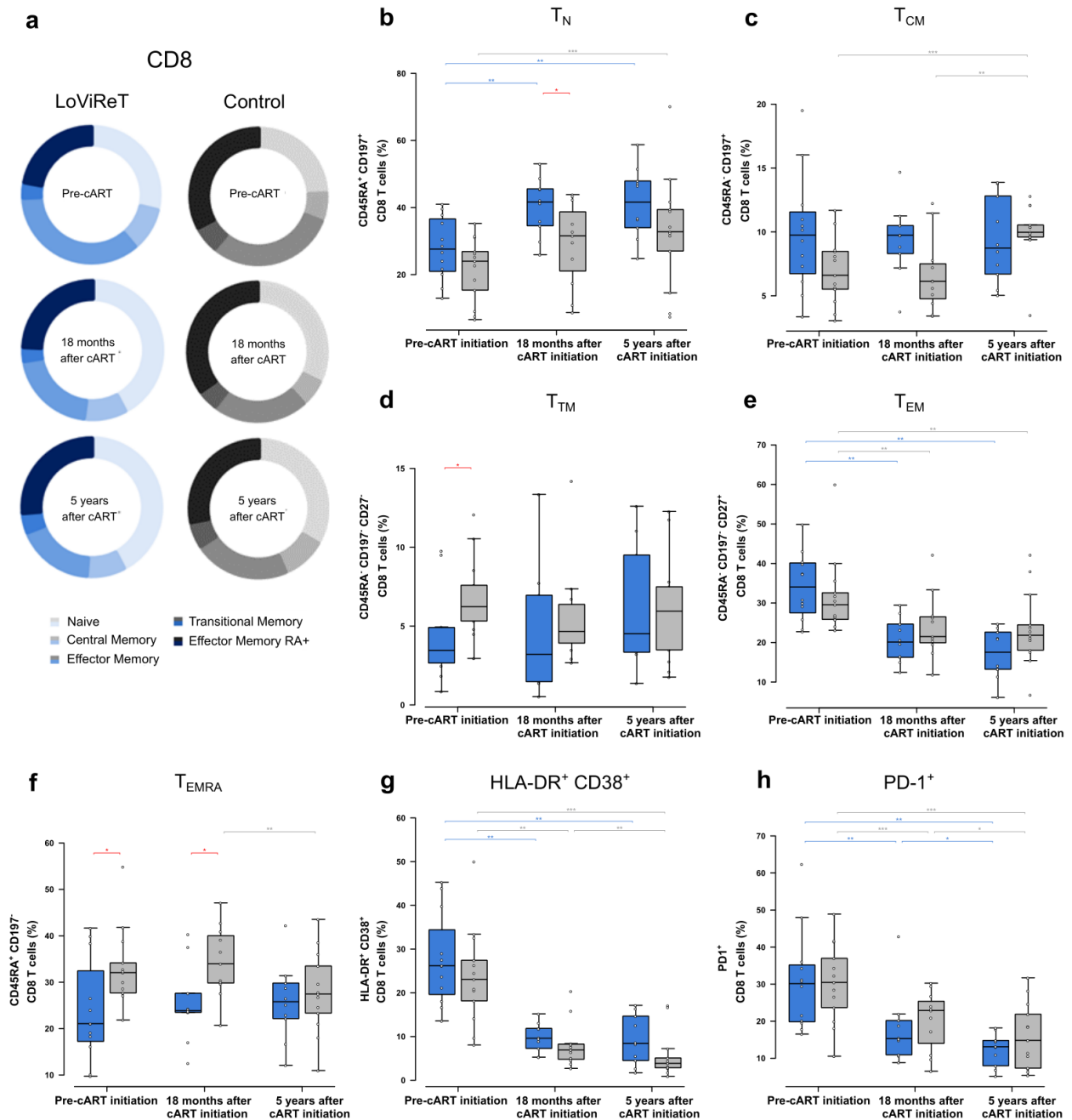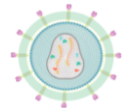
Figure 32. Analysis of maturation subsets, activation, exhaustion, and surrogate markers in the reservoir in CD8+ T cells. (**a**) Median CD8+ T-cell values for the frequency of the maturation subsets (naïve, central memory, effector memory, transitional memory, and effector memory RA+) in controls and cp-LoViReT at three different time points: pre-cART, 18 months after initiation of cART, and 5 years after initiation of cART. Maturation stages were defined based on the combination of CD45RA, CD197 (CCR7), and CD27. (**b**) CD45RA+CD197+ (Naive), (**c**) CD45RA−CD197+ (central memory), (**d**) CD45RA−CD197−CD27− (transitional memory), (**e**) CD45RA−CD197−CD27+ (effector memory), and (**f**) CD45RA+CD197− (effector memory RA+). (**g**) CD8 activation levels (HLA-DR+CD38+) in both study groups over time. (**h**) CD8+ T-cell exhaustion marker PD-1 (CD279). The cp-LoViReT group is depicted in blue and the control group in grey.

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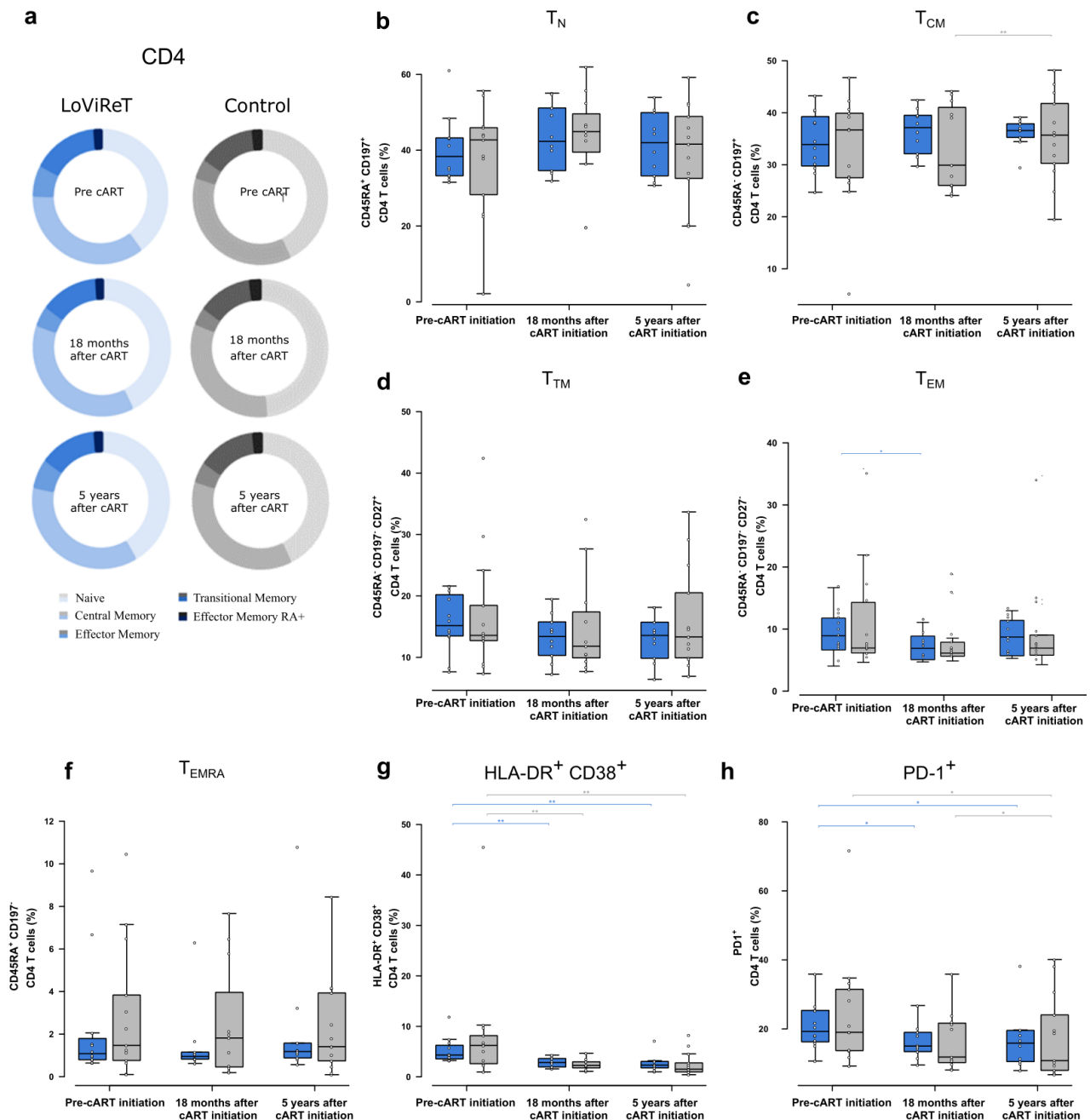
Figure 33. Analysis of maturation subsets, activation, exhaustion, and surrogate markers in the reservoir in CD4+ T cells. (**a**) Median CD4+ T-cell values for the frequency of the maturation subsets (naïve, central memory, effector memory, transitional memory, and effector memory RA+) in the control and cp-LoViReT groups at three different time points: pre-cART, 18 months after initiation of cART, and 5 years after initiation of cART. Maturation stages were defined based on the combination of CD45RA, CD197 (CCR7), and CD27. (**b**) CD45RA+CD197+ (naive), (**c**) CD45RA−CD197+ (central memory), (**d**) CD45RA−CD197−CD27− (transitional memory), (**e**) CD45RA−CD197−CD27+ (effector memory), and (**f**) CD45RA+CD197− (effector memory RA+). (**g**) CD4 activation levels (HLA-DR+CD38+) in both study groups over time. (**h**) CD4+ T-cell exhaustion marker PD-1 (CD279). The cp-LoViReT group is depicted in blue and the control group in grey.
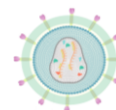
In summary, we observed changes in CD8$^+$ T-cell maturation in cp-LoViReT with higher levels of T$_N$ in combination with lower T$_{TM}$ and T$_{EMRA}$ CD8$^+$ T cells before initiation of cART. This observation was not associated with changes in activation or exhaustion of CD8$^+$ T cells in this group.

3b.3.5. CD4$^+$ susceptibility and viral inhibition by CD8$^+$ and NK cells

To evaluate the functionality of the T cells in both groups of individuals, we analyzed the susceptibility of the target cells to HIV-1 infection and viral inhibition by CD8$^+$ and NK cells in the same samples as those used for NK immunophenotyping. Thus, we selected 11 cp-LoViReT and 11 controls with available samples before initiation of cART and after 5 years on cART. We pulsed autologous CD4$^+$ T cells from each individual with a laboratory-adapted R5- viral strain for 7 days (Fig. 34a). We observed that CD4$^+$ T cells from cp-LoViReT and cp-controls were susceptible to HIV-1 infection and that there were no significant differences between groups before initiation of cART or after 5 years on cART.

Autologous CD8$^+$ T cells were also tested to analyze the suppression of viral replication (Fig. 34b), no significant differences between cp-LoViReT and controls were observed. High variability in the inhibition percentage was recorded in all the samples assayed, this could be explained by the limitation arising from the use of frozen cells in this assay.

Similarly, we did not find significant differences in the percentage of inhibition by autologous NK cells between groups before initiation of treatment or after 5 years on cART (Fig. 34c).

In conclusion, CD4$^+$ T cells from cp-LoViReT were perfectly susceptible to HIV-1 infection, with no signs of distinct CD8 and NK cytotoxic activities compared with control individuals.

a CD4⁺ T cell susceptibility to HIV infection

b Viral inhibition by CD8⁺ T cells

c Viral inhibition by NK cells
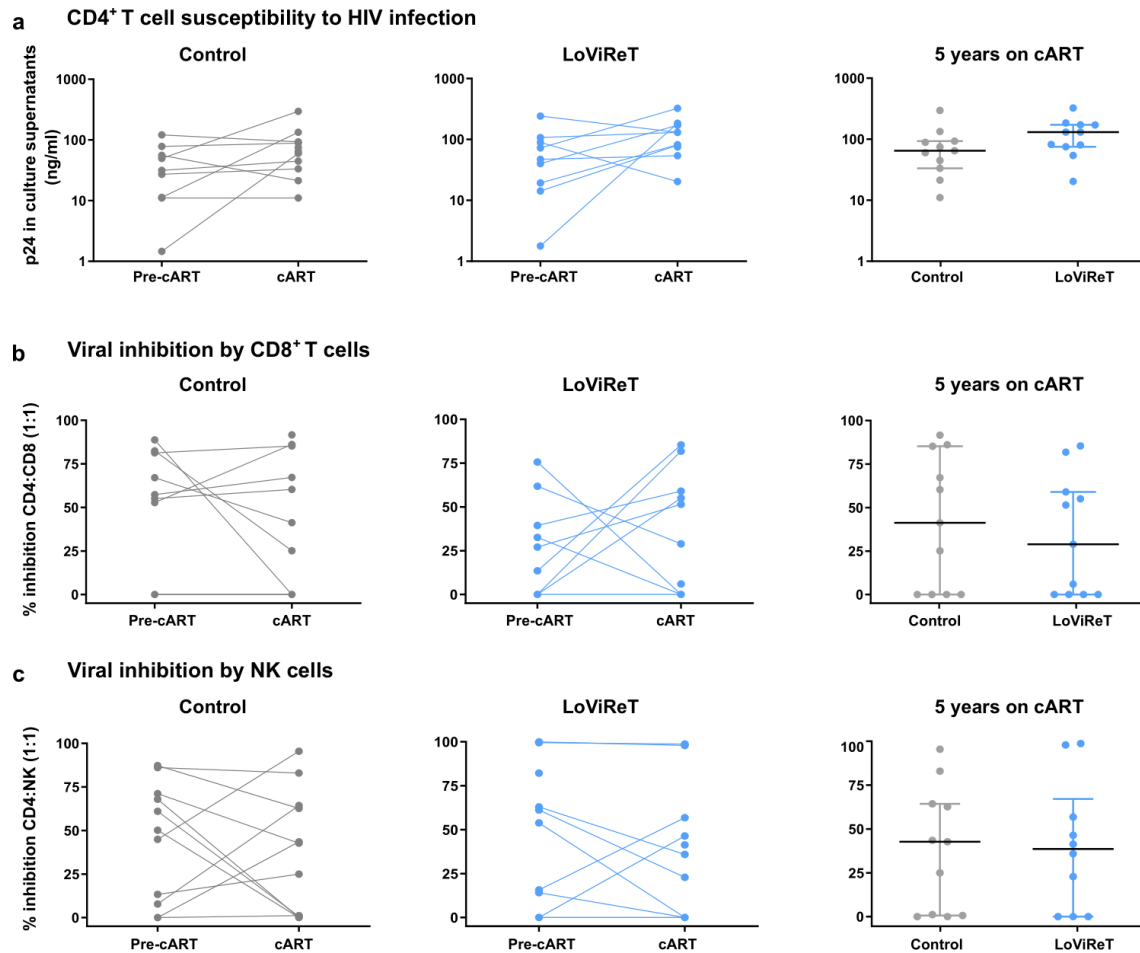
Figure 34. CD4$^+$ T cell susceptibility to HIV-1 and viral inhibition by CD8$^+$ T cells and NK cells. (**a**) PBMC samples before initiation of cART and after 5 years of cART were used to analyze the susceptibility of CD4$^+$ T cells to HIV-1$_{BaL}$ (CCR5 tropic strain) (10 ng p24/ml). We also measured the *ex vivo* ability of (**b**) CD8$^+$ T cells and (**c**) NK cells to inhibit superinfected autologous CD4$^+$ T cells at a 1:1 ratio. The cp-LoViReT group is depicted in blue and the control group in grey.

3b.3.6. Inflammatory marker levels in cp-LoViReT individuals

We measured soluble plasma pro-inflammatory or homeostatic cytokines (IL-2, IL-6, IL-7, IL-10, IL-27, IP10) and the coagulation factor D-dimer in plasma samples from eight cp-LoViReT and nine controls before cART and after five years on cART (selected according to sample availability). We did not find statistically significant differences either over time or between groups (Table 7).

Table 7. Inflammatory, homeostatic, and coagulation plasma biomarkers in cp-LoViReT and controls before and after cART.

| Parameter | PRE-ART | | | HAART | | |
| | Control | LoViReT | | Control | LoViReT | |
	Median [IQR]	Median [IQR]	P value*	Median [IQR]	Median [IQR]	P value*
IL27	4.9 [4.9 - 29.5]	50.4 [4.9 - 67.4]	0.100	15.7 [4.9 - 83.1]	4.9 [4.9 - 33.8]	0.453
IL2	107.5 [102.2 - 120.5]	121.9 [91.7 - 173.7]	0.838	114.8 [83 - 121.9]	107.5 [84.9 - 116.3]	0.539
IP10	64.1 [53.7 - 94.8]	59.6 [53.4 - 68.8]	0.497	35.3 [27.4 - 63.5]	30.9 [27.8 - 59.9]	0.720
IL6	4.7 [4.7 - 15.9]	4.7 [4.7 - 44.9]	0.887	4.7 [4.7 - 73.7]	4.7 [4.7 - 4.7]	0.255
IL7	5.6 [4.4 - 7.7]	5.6 [4.4 - 7.9]	1	7.3 [5.1 - 9.2]	6.7 [5.4 - 7.1]	0.347
IL10	5.3 [4.2 - 6.8]	8.2 [4.6 - 9.3]	0.540	5.7 [3.9 - 7.7]	6.6 [5.4 - 7.8]	0.774
D Dimer	8978.3 [5304.3 - 18544.5]	11486.3 [9243.1 - 14256.2]	0.968	5889.2 [4242.7 - 12242.4]	6933.2 [4292 - 8320.8]	0.842

*Wilcoxon rank sum test

3b.3.7. HIV-1 specific antibodies

To determine whether low reservoirs could be associated with humoral responses to HIV-1, we analyzed HIV-1 specific antibodies in plasma samples before initiation of cART and after five years on cART in eight cp-LoViReT and nine controls for whom plasma samples were available. We observed a decrease in HIV-1 specific antibody levels in both groups after initiation of cART (Fig. 35a). This decay was greater in cp-LoViReT than in controls (Fig. 35a).

We also measured the avidity of antibodies using a limiting antigen avidity assay. We observed a trend toward a more pronounced reduction in antibody avidity in cp-LoViReT when cART was initiated, with most individual levels falling below the cut off seen in acute HIV-1-infected individuals (Fig. 35b).

Therefore, HIV-1 antibody quantity and quality were more markedly diminished upon initiation of cART in cp-LoViReT.
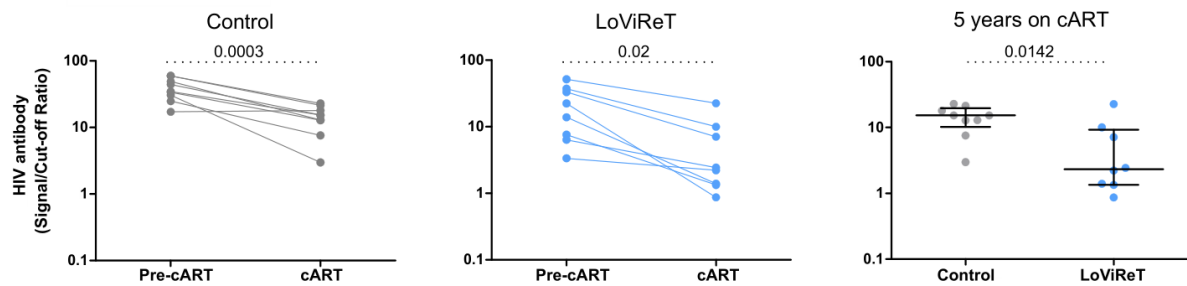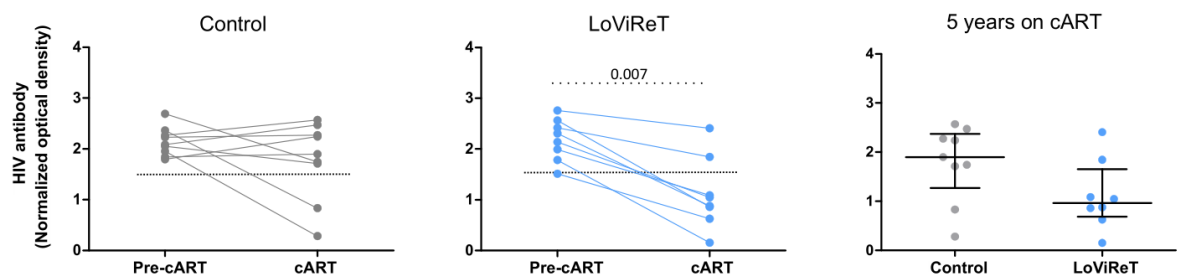
Figure 35. Measurement of HIV-1 specific antibodies. Plasma samples before initiation of cART and after 5 years of cART were tested for HIV-1 specific antibody levels using a (**a**) detuned version of the HIV-1 VITROS assay and a (**b**) limiting antigen avidity assay. The dotted line represents the HIV-1 antibody assay diagnostic cut-off level used to classify individuals as HIV-1-positive or -negative. The p-values between groups were assessed using the Mann-Whitney test.

3b.4. DISCUSSION

Latency in CD4$^+$ T cells is established very early after infection and is the major obstacle to curing HIV-1 infection[294]. A low HIV-1 DNA reservoir has been associated with early treatment[65], and better clinical outcome[295]. Nevertheless, the size of the HIV-1 DNA reservoir is not necessarily associated with time to viral rebound in individuals treated early after infection and who discontinued their cART[242,295]. The global frequency of HIV-1-infected individuals harboring low levels of HIV-1 DNA and the determinants of these levels remain unknown.

In our study, we found that 9.3% of the individuals screened had HIV-1 DNA levels below 50 copies/10^6 PBMCs (LoViReT individuals). The proportion of LoViReT individuals was similar in the two recruiting centers, indicating that our data might be extrapolated to other clinical sites with similar subject populations and viral subtypes. Previous studies in treated chronically infected individuals found that 28% and 19% of individuals had HIV-1 DNA levels below 150 copies/10^6 PBMCs and 66 copies/10^6 PBMCs, respectively[243,244]. While both studies revealed greater amounts of individuals with low HIV-1 DNA levels, the differences observed with our results could be explained by their higher cut-off for defining low HIV-1 DNA. Reanalysis of our dataset after applying their cut-offs revealed that 33% and 19% of individuals, respectively, had a "low reservoir", thus confirming this hypothesis. We maintained our cut-off of 50 copies/10^6 PBMCs because it represents the 10th percentile of the screened population.

Various clinical parameters were associated with LoViReT status, including a higher CD4 nadir, lower maximum pVL, and fewer detectable pVLs during cART. Nevertheless, the model was not strong enough to identify LoViReT individuals, suggesting that other viral or immunological factors might be involved. The same three factors have been found to be closely associated with harboring low levels of HIV-1 reservoir[243,244,296,297], probably because a higher CD4$^+$ T-cell nadir could prevent repopulation of the immune system with expanded latently infected lymphocytes. Moreover, a lower pVL peak might have prevented massive seeding of CD4$^+$ T cells by HIV-1. Similarly, fewer detectable pVLs during the course of the treated infection might have prevented replenishment of the reservoir.

Previous studies, have reported the association between early treatment and low viral reservoirs[298,299]. However, only individuals treated in Fiebig I-IV (between one week and one month after infection) were capable of achieving low levels of total HIV-1 DNA after more than three years on treatment[65,288]. Surprisingly, if early treatment is excluded as the main cause of a remarkably low reservoir, 66% of the LoViReT individuals were treated more than six months after acquisition of HIV-1 (cp-LoViReT). Since eradication strategies need to be effective in the vast majority of treated chronically infected individuals, it would be of considerable interest to know the mechanism responsible for the low reservoir detected in this group of individuals.

Data on the kinetics of HIV-1 reservoirs have been reported in treated chronically infected individuals[108,300,301] harboring standard levels of total HIV-1 DNA or treated during primary infection[116,288]. However, to our knowledge, this is the first study to address the kinetics of reservoirs in chronically HIV-1-infected individuals harboring low levels of total HIV-1 DNA, including the pre-cART period. Our results provide the first evidence that cp-LoViReT have intrinsically lower levels of total HIV-1 DNA before starting treatment, despite having the same levels of plasma viremia as controls. Besides, once cART is introduced, their reservoir seems to be more cART-sensitive, since decay of total HIV-1 DNA is faster in cp-LoViReT. We also proved that their CD4$^+$ T cells were susceptible to infection; therefore, the low reservoir observed is probably due to factors that do not severely impact the viral replication cycle in the cells. Hence, we cannot exclude the possibility that cp-LoViReT might have more linear unintegrated DNA, which decays faster than integrated DNA in the presence of cART[137,302]. Additionally, and consistent with other authors, we found a positive correlation between pre-treatment levels of total HIV-1 DNA and levels after five years on cART, thus demonstrating the importance of certain features before initiation of cART, such as host factors and/or the immune system, in determining subsequent reservoir size[67,289].

A naturally low level of cell-associated HIV-1 DNA could result in a more preserved immune system. cp-LoViReT had fewer CD8$^+$ T$_{TM}$ and T$_{EMRA}$ in the absence of cART, with frequencies similar to those of HIV-1-negative individuals, suggesting a less impaired CD8$^+$ T-cell compartment. HIV-1 infection disrupts T-cell

subset homeostasis, with a dramatic decrease in the frequency of CD8$^+$ T$_N$ cells and massive expansion of CD8$^+$ T$_{TM}$ cells in infected individuals treated during both the acute and the chronic phase[303]. Furthermore, after 18 months on therapy, the differences in CD8$^+$ T$_N$ cells between cp-LoViReT and controls become statistically significant, with the result that CD8$^+$ T$_N$ cells were more numerous than in controls. The higher proportion of CD8$^+$ T$_N$, also observed in ECs, may suggest shared preservation of thymic function[70]. However, in LoViReT individuals, the preserved CD8$^+$ T-cell compartment does not seem to be associated with an enhanced cytotoxic capacity of their CD8$^+$ T cells or NK cells, in contrast to HIV-1 controllers for CD8$^+$ T cells[304] and post-treatment controllers for NK cells[305]. Further exploration with fresh cells, more participants, and assessment of the frequency of HIV-1 specific cells will be needed to fully elucidate this point.

Finally, consistent with the kinetics of total HIV-1 DNA, the marked decline in the quantity and avidity of HIV-1 specific antibodies in cp-LoViReT during cART indicates a lower amount of circulating HIV-1 antigen. Our findings are in line with results in treated chronically infected individuals from Keating *et al*[306] who reported an association between declining antibody levels during cART and lower levels of antigen production, better viral control, and lower systemic viral burdens.

cp-LoViReT represent a new phenotype of individuals characterized by low intrinsic total HIV-1 DNA, better immune preservation, and low circulating HIV-1 antigens despite being treated in the chronic phase of the infection. Levels of proviral DNA while on cART in some individuals of the well-characterized post-treatment and EC cohorts were as low as those of LoViReT individuals, with median levels of 1.7 and 1.5 log$_{10}$ copies per million PBMCs, respectively[307]; however, we can rule out an overlap between cohorts. LoViReT had high viral loads during chronic infection before initiation of treatment. This finding differs from the main factors associated with elite and post-treatment controller cohorts. In addition, various studies suggest that having low amounts of HIV-1 DNA does not prevent early and consistent viral rebound if therapy is interrupted in infected individuals receiving long-term treatment[239–241]. Therefore, a very low reservoir is not necessarily associated with spontaneous control of viral replication, as might be the case in ECs or post-treatment controllers. However, when attempting to identify combined approaches

toward finding a cure for HIV-1 infection, cp-LoViReT appear to be excellent research participants, since they have better preserved immune cell populations and start from a smaller reservoir, which might be an advantage over individuals with a larger reservoir.

In conclusion, we found that 9% of the HIV-1-infected individuals have low levels of total HIV-1 DNA (LoViReT individuals) and that most were treated in the chronic phase of the infection. Moreover, they are characterized by a viral reservoir that is intrinsically reduced before initiation of cART and enhanced decay after initiation of treatment, suggesting an impaired HIV-1 reservoir establishment. Also, they have a less compromised CD8$^+$ T-cell compartment and lower HIV-1 specific antibody levels, probably as a result of lower amounts of circulating antigens. Additional studies will be necessary to unravel why these individuals have not reached an HIV-1 control as well as the nature of their reservoir.

Chapter 3c. LoViReT individuals show altered HIV-1 latency distribution

3c.0. PRESENTATION

The study in chapter 3b led us to stablish the cohort of LoViReT individuals, which showed that a 9.3% of the HIV-1-infected individuals harbor low levels of HIV-1 DNA. However, the study was only done in blood due to their retrospective design, and it was unknown if such a low reservoir could also be found in anatomic sanctuaries including rectum or lymph node, or if these proviruses were replicative. Moreover, it was not clear why these individuals did not control the virus similarly to EEC. A hypothesis was that HIV-1 specific immune responses were not as efficient as in EEC. Therefore, a prospective observational study was performed with a subgroup of the LoViReT individuals characterized in chapter 3b, regardless of when they started cART, and compared to HIV-1-infected individuals with a standard reservoir size.

3c.1. INTRODUCTION

The viral reservoirs have a high interindividual variability in the number of latently HIV-1 infected cells, despite having suppressed viremia. However, a small proportion of HIV-1-infected individuals harbor low reservoirs such as EC[238], EEC[308] (seen in Chapter 3a) and PTC[72] who are able to naturally control viral replication in the absence of cART. These phenotypes are mainly associated with viral and host factors. We have previously observed that in EEC (and also in EC) there is an enrichment in protective HLA alleles, potent cytotoxic CD8^{+} T-cell activity, and lack of viral evolution probably by the presence of defective provirus[263,308–310]. In contrast, PTC individuals are associated with early treatment, enrichment in risk HLA alleles, weak HIV-1 specific cytotoxic activity, and unusual HIV-1 latency distribution among CD4^{+} T cell subpopulations[72].

We and others had previously described a new phenotype of individuals, dubbed LoViReT, which harbor low level of total HIV-1 DNA under cART[311]. They accounted for 9% of all HIV-1-infected individuals and were mainly treated during the chronic phase of infection. This new phenotype is mainly characterized by a naturally reduced and more cART-sensitive viral reservoir, less impaired CD8^{+} T-cell compartment before cART, and low circulating HIV-1 specific antibodies. However, it is still not clear the underlying functionality of this low levels of provirus and whether this can also be observed in anatomical compartments, where the reservoir is usually higher.

In this study, we have comprehensively analyzed a subgroup of 22 LoViReT individuals in comparison with individuals with standard viral reservoir size, all under cART. Since here we have prioritized to include a larger number of individuals, both acute and chronically treated individuals are considered for this study. We have analyzed host factors, functional integrity of their provirus, levels of reservoirs in secondary immune tissues, and the distribution of provirus among different CD4^{+} T cell subpopulations.

3c.2. METHODS

3c.2.1. Participants and study design

From the 42 LoViReT individuals defined in Chapter 3b[311], we selected 22 LoViReT (both acute and chronically treated) which still met the inclusion criteria and the willingness to participate in the study. Also, we selected 22 controls. Participants were selected from Hospital Germans Trias i Pujol and Hospital Clinic of Barcelona (Spain). The inclusion criteria for both groups were to be under suppressive cART with undetectable viremia (HIV-1 RNA <50 copies/ml) for at least three years, and have <100 DNA copies/10^6 CD4$^+$ T cells for LoViReT and >100 HIV-1 DNA copies/10^6 CD4$^+$ T cells for controls. Groups were matched by clinical characteristics using random forest. Treated chronically infected participants were defined as individuals with more than six months between acquisition of HIV-1 and initiation of treatment.

In a second phase of the study, a subgroup of 14 individuals underwent a leukapheresis to obtain high numbers of cells to perform a qVOA and measure the viral distribution among CD4$^+$ T cell subpopulations. Then, those with an IUPM smaller than 0.1, and the willingness to participate, underwent rectum and lymph node biopsies. For ethical reasons, in control individuals we did not perform this second phase of the study (Fig. 36).

All individuals provided their signed informed consent to participate in the study. The study was approved by the Ethics Committee at both recruiting hospitals (reference #: PI-17-043).
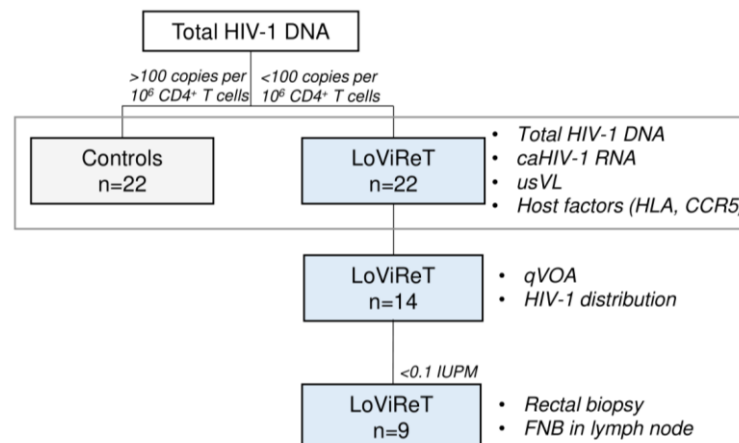
Figure 36. Study design flow-chart. usVL= ultrasensitive viral load; qVOA= quantitative viral outgrowth assay; IUPM= Infectious Units per Million; FNB= fine needle biopsy.
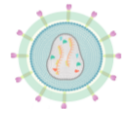
3c.2.2. Quantification of HIV-1 reservoir in blood

To evaluate the size of the viral reservoir, lysed extracts from CD4$^+$ T cells were used to measure total HIV-1 DNA, as previously described in chapter 3a.

Viral transcription was evaluated by quantification of caHIV-1 RNA in purified CD4$^+$ T cells, using one-step reverse-transcription ddPCR. The 5'-LTR or *gag* genes and the housekeeping gene *TBP* were measured in parallel. The residual viremia (<50 HIV-1 RNA copies/ml plasma) was measured by the usVL assay from 9 ml of plasma with a LOD of 0.56 copies/ml, as previously described in chapter 3a.

3c.2.3. HLA typing

High-resolution HLA class I typing for B alleles was performed by sequence-based typing methods. B*27 and B*57 alleles were considered protective, and B*35 and B*07 as risk alleles[263].

3c.2.4. *CCR5* genotyping

A portion of the *CCR5* gene was amplified by PCR with primers that flanked the 32-bp deletion[312]. Wild-type and deleted fragments of 185 bp and 153 bp, respectively, were generated and visualized in 2% agarose gels. Heterozygosity (*CCR5* wt/Δ32) was indicated by the presence of both fragments.

3c.2.5. Quantitative viral outgrowth assay

Leukaphereses were obtained from 14 LoViReT to quantify the size of the replication-competent reservoir. We performed a limiting dilution cell culture assay with 38 million of CD4$^+$ T cells with the detection limit set at 0.0185 IUPM, as previously described in chapter 3a.

3c.2.6. Quantification of HIV-1 reservoir in anatomical compartments

Total HIV-1 DNA was measured in anatomical compartments from secondary immune tissues, including rectal biopsies and LN in LoViReTs with an IUPM lower than 0.1. For rectal biopsies, four to eight endoscopic biopsies per individual were collected from rectum using 2.5 mm forceps, which were immediately placed in complete medium (RPMI 1640 with 10% FBS (both from Gibco), supplemented with antibiotics (500 μg/ml piperacillin/tazobactam, Fresenius Kabi) and 1.25 μg/ml amphotericin B (Fungizone, Gibco)). Samples were processed immediately after

collection in order to minimize loss of lamina propria leukocytes (LPL) and/or bacterial contamination. Biopsies were incubated in HBSS (without Ca^{2+}/Mg^{2+}, Gibco) containing 1 mM DTT (Sigma) and 1 mM EDTA (Gibco) for 25 min, at RT with constant shaking, to remove the epithelial layer. Then, biopsies were transferred to complete media and cultured overnight in 6-well low-binding plates (Costar). Culture supernatants were collected to recover the released cells, and the remaining tissue was disrupted by gentle pipetting. Finally, cell suspensions were cleared from tissue debris using a 40 μm nylon mesh (Falcon). $CD45^+$ cells from the LPL were sorted in a BD FACSAria II Flow cytometer using the following antibodies: CD45 (FITC), CD3 (APC) and CD8 (PercP).

Regarding LN biopsies, a non-invasive technique scanner-guided Fine Needle Biopsy (FNB) was used, to aspirate cells from punctures of two inguinal LNs. Cells were placed in RPMI 1640 containing 1% Penicillin/Streptomycin and 10% FBS. Memory $CD4^+$ T cells defined as $CD3^+CD4^+CD45RA^-$ were sorted in a BD FACSAria II Flow cytometer using the following antibodies: CD3 (APC), CD8 (APC H7), CD45RA (FITC).

In both cases, isolated cells were lysed, and total HIV-1 DNA was quantified by ddPCR as previously described.

3c.2.7. Cell sorting of $CD4^+$ subpopulations

$CD4^+$ T cell subpopulations were isolated by cell sorted from leukaphereses obtained from 14 LoViReTs, starting from 175-450 million of PBMCs. PBMCs were incubated with live/dead (APC-Cy7), CD3 (BV510), and CD4 (AF700) monoclonal antibodies. T-cell maturation was based on the expression of the surface markers CD45RA (APC), CCR7 (PE Dazzle) and CD27 (FITC) in order to define T_N ($CD45RA^+CCR7^+CD27^+$), T_{CM} ($CD45RA^-CCR7^+ CD27^+$), T_{TM} ($CD45RA^-CCR7^- CD27^+$), T_{EM} ($CD45RA^-CCR7^-CD27^-$), and T_{TD} ($CD45RA^+CCR7^-CD27^-$). Subpopulations were sorted in BD FACSAria II Flow cytometer, and cells were lysed to measure total HIV-1 DNA by ddPCR.

3c.2.8. Statistical analysis

The clinical characteristics of the study population were presented as percentages for categorical variables and as median and IQR for continuous variables. Differences between LoViReT controls or standard individuals were assessed using the Mann-Whitney test in the case of continuous variables and the Fisher exact test in the case of categorical variables. The analyses were performed with GraphPad (v8.02).

3c.3. RESULTS

3c.3.1. Participant characteristics

We selected 22 LoViReT and 22 controls with more than three years under cART and fulfilling inclusion criteria. Their clinical characteristics are summarized in Table 8. No statistically significant differences were observed between LoViReTs and controls. The clinical histories of LoViReT individuals confirmed that 14 LoViReT were treated in the chronic phase of the infection (>six months since acquisition). Out of the remaining LoViReT individuals, five were early treated, and three lacked proven information of the time being infected at initiation of cART. Therefore, the proportion of LoViReT treated in the chronic phase of infection are comparable to what we observed in our previous study (Chapter 3b).
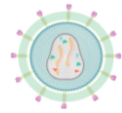
Table 8. Clinical characteristics of the individuals included in the study

	LoViReT group (n=22)	Control group (n=22)	p-value
Age at diagnosis (years), median [IQR][a]	34 [28-39]	33 [30-37]	0.7
Sex (females), n (%)[b]	5 (23)	2 (9)	0.22
Mode of infection			
MSM, n (%)[b]	10 (45)	12 (55)	
Heterosexual, n (%)[b]	7 (32)	5 (23)	0.52
IVDU, n (%)[b]	3 (14)	3 (14)	
Time since diagnosis (years), median [IQR][a]	17 [10-23]	20 [12-27]	0.52
Time in cART (years), median [IQR][a]	13 [9-18]	17 [11-25]	0.12
Time with supressed VL (years), median [IQR][a]	12 [9-16]	13 [11-16]	0.34
Zenith (log_{10} copies/ml plasma), median [IQR][a]	4 [3.5-4.9]	4.6 [3.6-5.1]	0.46
$CD4^+$ T cells			
Nadir (cells/µl), median [IQR][a]	300 [235-439]	297 [219-420]	0.58
Actual CD4 count (cells/µl), median [IQR][a]	859 [527-984]	787 [573-1025]	0.86

IQR= interquartile range; MSM= men who have sex with men; IVDU= intravenous drug users; VL= viral load.

3c.3.2. LoViReT individuals lacked host protective factors

In order to determine if the low levels of total HIV-1 DNA could be associated with host genetic factors, we first measured the HLA class I alleles to determine if LoViReT individuals have a different pattern of protective/risk alleles than controls with standard HIV-1 DNA. Six LoViReTs had protective alleles (two HLA-B*57 and four HLA-B*27) while only two controls had protective alleles, but the differences were not statistically significant compared to controls (p=0.27). However, although we did observe a lower frequency of risk alleles (HLA-B*07 and B*35) in the LoViReT

group than in controls, the differences were not statistically significant between groups (p=0.07) (Fig. 37a).
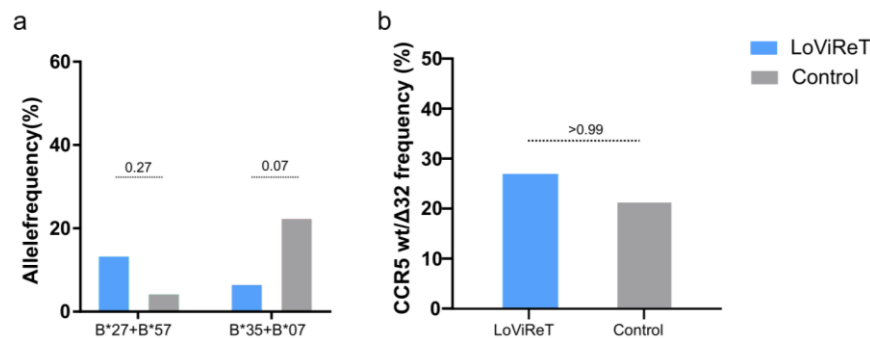
Figure 37. HLA class I profile and *CCR5* wt/Δ32 frequency. (**a**) The frequencies of the protective alleles HLA-B*27 and B*57, the risk alleles HLA-B*07:02 and B*35 and the frequency of the genotype *CCR5* wt/Δ32 in LoViReT and controls. (**b**) Frequency of individuals with the *CCR5* Δ32 mutation in heterozygosis.

We also evaluated the frequencies of the *CCR5* Δ32 mutation among the cohort. We found that 27.3% of LoViReT individuals had a *CCR5* wt/Δ32 genotype while controls 21.4%, but this difference was not statistically significant (p>0.99) (Fig. 37b).

Thus, the low reservoirs observed in LoViReT does not seem to be associated with an increase in protective or risk host factors.

3c.3.3. LoViReT individuals have a lower HIV-1 DNA and HIV-1 RNA expression

Levels of total HIV-1 DNA were measured in CD4$^+$ T cells and compared with 22 controls with standard HIV-1 DNA, matched by clinical factors to confirm previous results from Chapter 3b in a more recent sample. As expected by experimental design, we observed statistically significant differences between groups (p<0.0001) (Fig. 38a).

Moreover, in order to determine if the low levels of total HIV-1 DNA were associated with a low HIV-1 expression, we analyze the levels of intracellular and extracellular forms of HIV-1 RNA by measuring caHIV-1 RNA and usVL in plasma, respectively, and compared with controls. LoViReT had lower levels of caHIV-1 RNA (p<0.0001) despite of all individuals were detectable for HIV-1 expression (Fig. 38b).
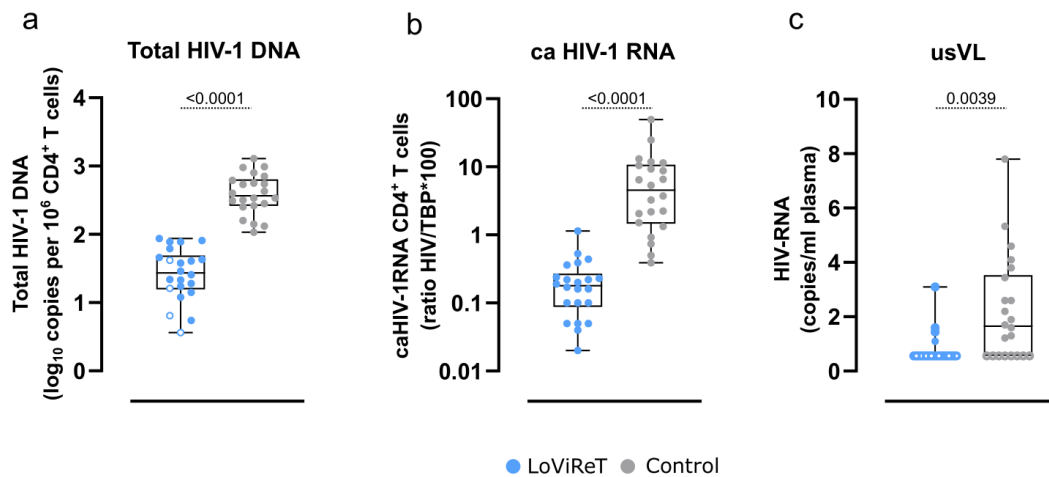
Figure 38. Measurements of total HIV-1 DNA and HIV-1 expression. (**a**) Levels of total HIV-1 DNA, (**b**) cell associated HIV-1 RNA and (**c**) ultrasensitive viral load in plasma. Open symbols represent values under the limit of detection, in those cases the limit of detection varied based on sample/volume input. usVL= ultrasensitive viral load.

Residual plasma viremia was detected in 59% of the individuals assessed. Interestingly, we observed a higher percentage of individuals under the LOD (0.56 copies/ml) in the LoViReT group (81%) than in controls (36%), and these differences were statistically significant (p=0.0039) (Fig. 38c).

Thus, the LoViReT phenotype is not only determined by a low HIV-1 DNA but also by a low expression of intracellular and extracellular HIV-1 RNA under cART.

3c.3.4. No differences in HIV-1 persistence in LoViReTs treated in the chronic or early phase of infection

In order to decipher if HIV-1 persistence in LoViReT is different depending on the time of treatment initiation, we analyzed the reservoir levels and HIV-1 expression by the moment of initiation of treatment in 19 LoViReT individuals of which we had confirmed clinical history of the time of treatment initiation. We had two groups including chronic treated LoViReT (>6 months since HIV-1 acquisition and initiation of cART) and acute treated LoViReT (<6 months since HIV-1 acquisition and initiation of cART). We did not find statistically significant differences in peripheral blood reservoirs, including total HIV-1 DNA (p=0.98), caHIV-1 RNA (p=0.81), and usVL (p=0.53) (Fig. 39a-c).
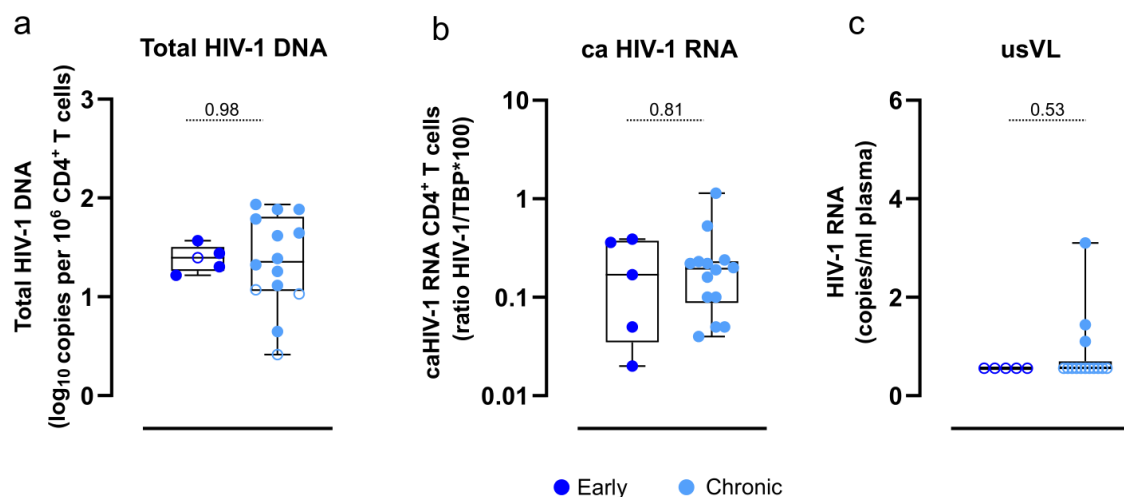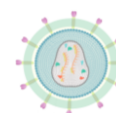
Figure 39. Measurements of total HIV-1 DNA and HIV-1 expression in LoViReT individuals treated in the chronic or early phase of HIV-1 infection**. (a)** Levels of total HIV-1 DNA, (**b**) cell associated HIV-1 RNA and (**c**) ultrasensitive viral load in plasma. Open symbols represent values under the limit of detection, in those cases the limit of detection varied based on sample/volume input. usVL= ultrasensitive viral load.

Since no differences were observed between LoViReTs treated in the chronic or early phase of the HIV-1 infection, the following analysis involving more invasive techniques were performed with both groups indistinctively.

3c.3.5. Low rate of replication competent virus in peripheral blood in LoViReT

To determine whether the low levels of provirus present in LoViReT individuals were replication competent, we performed a leukapheresis in a subgroup of 14 LoViReTs to obtain a large amount of CD4$^+$ T cells to perform a qVOA assay with a low limit of detection. We observed very low levels of replication competent virus, with 71% of the individuals having an IUPM under the LOD (0.0185), despite the high sensitivity of the assay. The four LoViReT with detectable qVOA had values ranging from 0.244 to 0.65 IUPM.

If we compare the IUPM values from LoViReT individuals with values from historical standard individuals under cART[264], we observed that LoViReT had lower statistically significant IUPM (p<0.0001) (Fig. 40a).

Moreover, we also did not observe differences in the proportion of LoViReT individuals with detectable IUPM if they were chronic or early treated, with 16.7% and 40%, respectively (p=0.55).

3c.3.6. Low viral reservoirs in secondary immune tissues in LoViReTs

We measured total HIV-1 DNA in rectum and LN biopsies in a subset of 8 LoViReT that had an IUPM below <0.1. In rectum, we detected levels of total HIV-1 DNA in 6/8 individuals with a median of 56.9 HIV-1 DNA copies/10^6 CD45$^+$ T cells [37.4-113.7], which is nearly 10-fold lower in comparison with historical values from standard individuals under cART (p=0.0004) (unpublished data from Bernal-Santateresa *et al*) (Fig. 40b).

Besides, in LN only 3 out of 8 individuals had detectable HIV-1 DNA with a median of 262.6 HIV-1 DNA copies/10^6 CD45RA$^-$ T-cells [36.3-2112]. In this case, the median value of HIV-1 DNA of LoViReT individuals did not differ much from historical standard individuals under cART[80]. However, this could be due by the fact that 63% of them were under the LOD, probably by the low number of cells we were able to obtain (Fig. 40c).

We also analyzed if there were differences in the reservoir in anatomical compartments between the LoViReTs treated in the chronic or early phase of the infection. No statistically differences were observed in the median levels of total HIV-1 DNA in rectum (p=0.19) or lymph node (p=0.23) between both groups.

Therefore, LoViReT individuals seem to have a lower or even undetectable reservoir compared to standard individuals under cART not only in peripheral blood but also in anatomical compartments such as rectum and LN, regardless when they started cART.
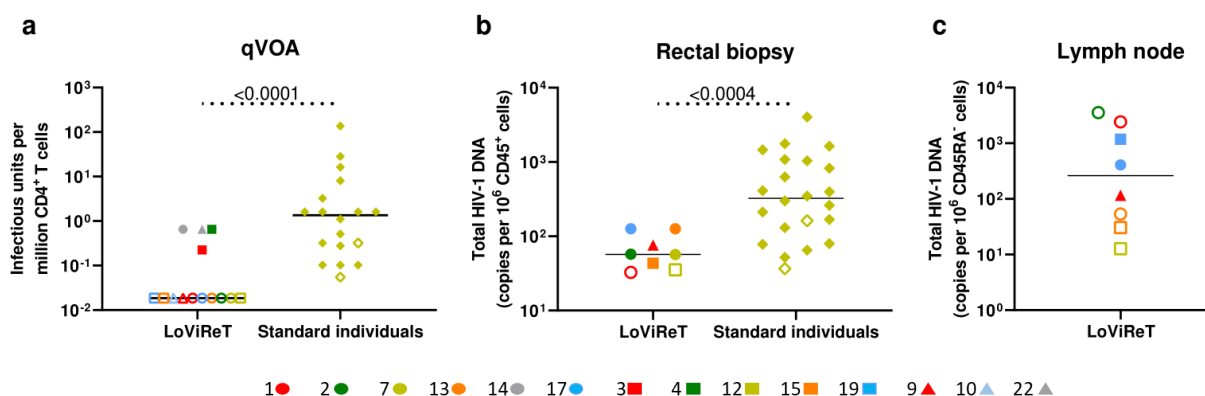
Figure 40. Measurements of the viral reservoir in peripheral blood and anatomical compartments. The figure shows data on each LoViReT individual for (**a**) viral outgrowth, (**b**) rectal and (**c**) LN biopsies compared with HIV-1-infected individuals with standard HIV-1 reservoirs. LoViReTs treated in the chronic phase of infection are represented by a circle, LoViReT treated in the early phase of infection are represented with a square and the ones with no available information
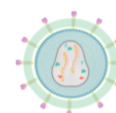
of their status when they initiated cART are represented with a triangle. HIV-1 DNA in rectal CD45$^+$ and LN CD45RA$^-$ were only assessed in LoViReTs with an IUPM <0.1. The values from standards individuals are from Morón-López et al[264] for the viral outgrowth, and from Bernal-Santateresa et al (unpublished data) for rectal biopsies. Median values are indicated by a horizontal black line. Open symbols represent values under the limit of detection, in those cases the limit of detection varied based on cell input. qVOA= viral outgrowth assay.

3c.3.7. High contribution of the short-lived CD4$^+$ T cells subpopulations to the HIV-1 reservoir in LoViReTs

To determine if the low level proviruses observed in LoViReT individuals could be attributed to the HIV-1 persistence in a specific CD4$^+$ T cell subpopulation, we sorted five CD4$^+$ T cell subpopulations from 14 LoViReTs (T$_N$, T$_{CM}$, T$_{TM}$, T$_{EM}$ and T$_{TD}$) and quantified total HIV-1 DNA in each population. The median levels of total HIV-1 DNA were 15, 51, 77, 79, and 165 copies/10^6 cells in the T$_N$, T$_{CM}$, T$_{TM}$, T$_{EM}$ and T$_{TD}$, respectively (Fig. 41a). Interestingly, T$_N$ and T$_{TD}$ had a high frequency of undetectable values, being 86% and 64% of the tested samples under the LOD, respectively.

To calculate each subset contribution to the HIV-1 reservoir, we evaluated the frequency of each subpopulation among CD4$^+$ T cells. The contribution of each subset to the reservoir showed that the major contributors to the total HIV-1 reservoir were T$_{TM}$ and T$_{EM}$, with median contributions of 47% and 29% respectively. Nevertheless, T$_N$ and T$_{TD}$ presented a very limited contribution to the HIV-1 reservoir with a neglectable contribution in both cases (Fig. 41b).
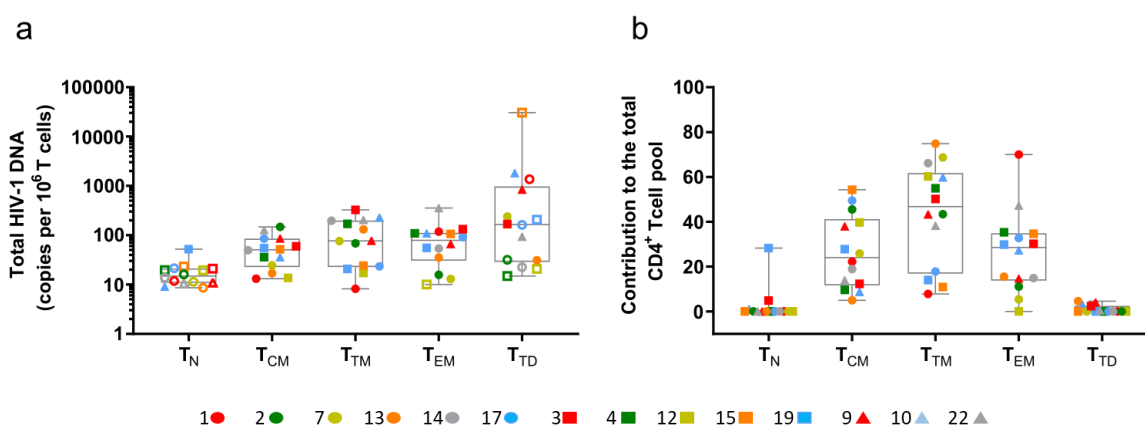
Figure 41. HIV-1 latency distribution in T-cell subsets in LoViReT individuals. (**a**) Total HIV-1 DNA measured by ddPCR and (**b**) contribution of each subset to the HIV-1 reservoir. LoViReTs treated in the chronic phase are represented by a circle, LoViReT treated in the early phase are represented with a square and the ones with no available information of their status when they initiated cART are represented with a triangle. Open symbols represent undetectable values, where the limit of detection is represented.

3c.4. DISCUSSION

We and others have previously described a new phenotype of HIV-1-infected individuals that harbor low levels of HIV-1 DNA in peripheral blood[239,241,243,311], which we called the LoViReT cohort. More than half of them were treated in the chronic phase of the infection and seem to have a better preserved CD8^{+} T-cell compartment. Here, we studied 22 LoViReT, regardless when they started cART, with a very low viral reservoir in peripheral blood, to explore the nature of the small HIV-1 latency level. Host factors, viral expression, defects in the viral replication-competence, latency in anatomical compartments and distribution among different peripheral cell subpopulations were studied.

We showed that the low amount of provirus present in LoViReT individuals were less transcriptional active in comparison with individuals with standard total HIV-1 DNA. And the low transcription lead to a less production of new viral proteins to form new virions, as we observed by the higher undetectability in residual viremia. This is in line with data from individuals with low levels of HIV-1 DNA such as ECs[238], EECs[308] and PTCs[72] where significant lower caHIV-1 RNA and residual viremia are observed.

Moreover, the low levels of HIV-1 expression found in peripheral blood were not able to effectively replicate in new cells since we observed that upon *ex vivo* stimulation of a high number of peripheral CD4^{+} T cells, a 71% of LoViReT did not show replication competent provirus. This result was also observed in a recent study where low replication-competent provirus were found in individuals with limited reservoir, however in that case all participants had low but detectable IUPM[241]. This may suggest that the low levels of proviruses found in peripheral blood detected by total HIV-1 DNA could be highly defective viral sequences since they had limited capacity to transcribe viral mRNA, generate viral particles or replication competent virions in peripheral blood under cART.

Alternatively, it has been widely described that HIV-1 reservoirs are 5-12 times larger in tissue compartments such as GALT[98,99] and LN[104] than in peripheral blood. To our knowledge, this is the first study to address the levels of HIV-1 DNA in rectum and LN in this kind of individuals with very low levels of reservoir in peripheral blood.

Our results showed that LoViReT individuals had limited provirus in rectum and LN, being in some cases even under the limit of detection. Therefore, this demonstrates that in these individuals, the cells with HIV-1 provirus are not preferentially residing in rectum or LN as happen with standard HIV-1-infected individuals.

Interestingly, we found that the time of initiation of treatment does not affect the levels of provirus, neither in peripheral blood nor in anatomical compartments. This is the opposite of what have been described in standard HIV-1-individuals where the earlier the treatment is started the lower is the reservoir[65,288]. Moreover, as we had previously shown LoViReT individuals already have special characteristics before initiation of treatment such as lower reservoir and better immune preservation[311]. Thus, the LoViReT phenotype seems to be defined by other factors, independent of treatment initiation.

One factor that might explain the total HIV-1 DNA dynamics observed in chapter 3b is the distribution of the provirus in the CD4$^+$ T cell subpopulations. LoViReT had a high contribution of the short-live T_{TM} and T_{EM} cells in the total HIV-1 reservoir, which are mainly composed of unstable viral forms and are easier to be eliminated through cART[313]. This might explain the more rapid decay of total HIV-1 DNA in LoViReT when they started cART[311]. In contrast, in the general HIV-1-infected population the latent reservoir is predominantly found in the long-lived cells such as T_{CM}[75], while in LoViReT individuals long-lived CD4$^+$ T cells, including T_N and T_{CM}, provided only 24% to the contribution to the total HIV-1 reservoir. This limited contribution of T_{CM} is also found in the classical sooty-mangabey model of attenuated SIV infection related to low CCR5 co-receptor expression[314], in HIV-2 infection where the virus is less pathogenic and replicative[315], in PTC[72] ,and also in LTNP associated with protective HLA-B*27 or B*57 alleles[316].

Since the overrepresentation of protective HLA-class I alleles in LTNPs[316], ECs[263,309,310] and EECs[308] and risk alleles in PTC[72] is associated with an atypical reservoir contribution and HIV-1 control, we checked this host factor in our cohort. We found that LoViReT had slightly higher frequency of protective alleles but without statistically significant differences. In contrast, other studies with similar individuals than LoViReT showed an enrichment in HLA protective alleles. However the

protective alleles in combination with a low reservoir was not enough to control viremia after treatment interruption[239]. Therefore, alternative factors at the transcriptome or protein level among others, might be involved in the protection of T_{CM} infection which in turn might have helped to attain some degree of control in the HIV-1 latency establishment.

Altogether, LoViReT share some characteristics with HIV-1 phenotypes of control such as EEC, EC and PTC but, as reported by other studies with similar phenotypes, are not able to control HIV-1 replication if cART is interrupted[239–241]. Nevertheless, their low reservoir stablished in subpopulations with a shorter half-life, which have an extremely low proliferative capacity[317], are less replication-competent[318], and have higher reactivation capacity upon treatment with some LRA[319] might be an important advantage when trying new eradicating strategies aimed at eliminating the viral reservoirs.

In conclusion, we found that the factors associated to the LoViReT status are related to an impaired reservoir establishment among different compartments with an altered proviral distribution in CD4+ T-cell subpopulations, which might generate a limited replicative capacity due to the protection of CD4+ T cell subpopulations with a long life span. The fact that LoViReT are not enriched in protective immunogenetic host factors seems to jeopardize the possibility of control HIV-1 replication in the absence of cART. However, their low reservoirs might be an advantageous characteristic for being candidates to HIV-1 eradication strategies. Those strategies might be oriented to mimic a strong immune system that can effectively eliminate the low amount of HIV-1 reservoir present in these individuals.

Chapter 3d. Mechanisms that contribute to a profound reduction of the HIV-1 reservoir after allogeneic stem cell transplant

This chapter corresponds to the manuscript:
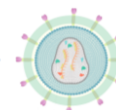
<u>Author's contributions</u>: The author of this thesis contributed to the current work by measuring ultrasensitive viral load, quantitative viral outgrowth, total HIV-1 DNA in anatomical compartments, humoral immune responses, and humanized mouse viral outgrowth assay. The author also contributed to the analysis and interpretation of the results and writing the manuscript.

3d.0. PRESENTATION

The previous chapters analyzed the different factors that can be associated to HIV-1 reservoirs that are naturally low. This chapter evaluates alternative factors associated to the diminution of HIV-1 reservoirs by therapeutic interventions. Specifically, we have studied the intrinsic effect of allogeneic hematopoietic stem cell transplant to reduce latency, since this approach has been the only one so far to prove a dramatic reduction of the HIV-1 reservoirs.

3d.1. INTRODUCTION

Allogeneic hematopoietic stem cell transplant (allo-HSCT) has contributed to the only known case of complete HIV-1 eradication (the "Berlin patient"). The underlying biological mechanisms are not fully understood, although the use of a donor with a homozygous mutation in the HIV-1 co-receptor CCR5 seemed to be key to preventing HIV-1 infection of the graft[229,320]. Other contributing factors may have been the conditioning regimen, which destroyed some or all reservoir T cells; an immunologic milieu favoring T-cell activation and reactivation of latent HIV-1; greater effectiveness at blocking reactivated virus spread by *CCR5*-mutated donor cells compared with suppressive cART; and alloreactivity that could have eliminated infected cells in the recipient[321].

However, transplant using *CCR5* wild-type donors also leads to a greater reduction in the latent reservoir than is obtained with any other clinical intervention[231,322–324]. For example, despite the delayed viral rebound after interruption of cART that was observed in two HIV-1-infected individuals undergoing allo-HSCT from *CCR5* wild-type donors (the "Boston patients")[325], these cases showed that allo-HSCT by itself was able to achieve large reductions in the viral reservoir. Transplant-associated mechanisms that reduce HIV-1 latency and thus may play a role in eliminating the virus need to be understood to allow development of less invasive strategies to eradicate HIV-1 infection that may be applicable to the broader population of HIV-1-infected individuals without hematologic disorders requiring stem cell transplant.

However, the scant experience with allo-HSCT in HIV-1-infected individuals prevents definitive conclusions[326]. To address that, the IciStem Consortium (www.icistem.org) has assembled the largest and most exhaustive observational cohort for the study of HIV-1 reservoir dynamics in HIV-1-infected individuals with hematologic disease and have undergone an allo-HSCT. The main objective is to evaluate the mechanisms responsible for the dramatic reduction in HIV-1 reservoirs associated with allo-HSCT.

In this study, we first evaluate the effect of the transplant to eradicate the infection analyzing the HIV-1-infected individuals included in the IciStem cohort that

have undergone an allo-HSCT. Then, we extensively studied six individuals from the cohort with the longest survival and follow-up (>2 years after allo-HSCT) who underwent allo-HSCT from *CCR5* wild-type donors. We analyzed the factors associated with allo-HSCT that are associated to the reduction of HIV-1 latency and viral-specific humoral responses. All this, in the absence of confounding HIV-1 resistance factors, such as *CCR5* Δ32 mutation.
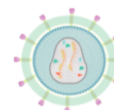
3d.2. METHODS

3d.2.1. Study participants

To date, the IciStem Cohort includes 40 individuals, where 30 had an exhaustive follow-up with a longitudinal sample collection. The patients were included in eight different countries: Spain, Netherlands, Germany, Switzerland, Belgium, Canada, UK, and Italy.

From the 30 individuals, twelve died within 2 years after transplant. From the remaining 18 individuals, 13 individuals survived more than 2 years after transplant, including three with *CCR5* Δ32 donor. Therefore, in the extensive study with individuals with a follow-up >2 years after allo-HSCT with a *CCR5* wild-type donor we selected six participants (IciS-01[327], IciS-03, IciS-06, IciS-17, IciS-27, and IciS-28). These individuals maintained the use of cART and achieved remission of their hematologic disease.

All participants provided informed consent. The observational protocol (IciStem study) was approved by the institutional ethical review boards in each country.

3d.2.2. Chimerism analysis

In four participants (IciS-01, IciS-03, IciS-06, and IciS-17), analyses were performed in whole BM, peripheral blood, or both. In three participants (IciS-01, IciS-03, and IciS-06), T cells and myeloid cells were purified from peripheral blood by immunomagnetic means (autoMACS, Miltenyi Biotec) using antibodies against CD3$^+$ and CD13/CD33$^+$, respectively. The minimum purity of isolated leukocyte subsets was 95%. In the other two participants (IciS-27 and IciS-28), mononuclear lymphocytes and monocytes were isolated, and the minimum purity was also 95%. In all participants, conventional chimerism analysis was performed with PCR of short tandem repeats (STR-PCR). In IciS-01, IciS-03, and IciS-06, when conventional chimerism analysis (with a sensitivity of 1%) was complete, ultrasensitive chimerism analysis in whole peripheral blood was also performed (Mentype DIPscreen and Mentype DIPquant, Biotype), with a sensitivity of 0.01% to 0.001%, depending on the quality and quantity of purified DNA. Complete chimerism was defined as the

absence of recipient-specific allelic patterns detectable by STR-PCR, with the level of sensitivity mentioned earlier.

3d.2.3. Quantification of HIV-1 reservoir in blood

HIV-1 DNA in PBMCs or bulk CD4+ T cells was repeatedly measured after allo-HSCT in each participant, as previously described in chapter 3a. Residual viremia (HIV-1 RNA) was also measured from 9 ml of plasma as previously described in chapter 3a. Leukaphereses were obtained from all participants in order to measure the number of infectious units in a large number of CD4+ T cells (range, 11 to 137 × 10^6 CD4+ T cells) as previously described in chapter 3a with the detection limit set at 0.005 IUPM.

3d.2.4. Quantification of HIV-1 reservoir in anatomical compartments

Per protocol, HIV-1 was measured in tissue biopsy specimens only in participants who had undetectable viral reservoirs in peripheral blood. Target cells for HIV-1 infection were isolated from different tissues to increase sensitivity for viral detection. CD45+ cells were isolated and processed from ileal biopsy specimens using the lamina propria leukocytes viral DNA assay, as previously described in chapter 3c[97]. T_{FH} CD4+ memory T cells, defined as CD3+CD4+CD45RA-PD-1+CXCR5+, were sorted by flow cytometry from LN biopsy specimens obtained using fine-needle aspiration, as previously described in chapter 3c. Magnetic cell isolation of CD3+ or CD4+ T-cell populations was performed in BM. In all cases, isolated cells were lysed, and viral DNA was quantified by ddPCR with two different sets of primers (table 2).

Lumbar puncture was performed to obtain 2 to 5 ml of CSF, and residual viremia was quantified[320].

3d.2.5. Quantification of HIV-1 antibodies

Specific HIV-1 antibodies in longitudinal plasma samples were measured using a qualitative Western blot assay (New LAV Blot I, Bio-Rad) and the quantitative standard and low-sensitivity versions of the VITROS anti-HIV-1 assay (Ortho Clinical Diagnostics)[261], as previously described in chapter 3a.
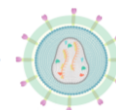
3d.2.6. Humanized mouse Viral Outgrowth Assay

As an in vivo measure of residual replication competent reservoir cells in blood, we used a humanized mouse model modified to transfer CD4$^+$ T cells instead of total peripheral blood mononuclear cells[168]. All procedures were performed according to protocol 8927, which was reviewed by the Animal Experimentation Ethics Committee of the University Hospital Germans Trias i Pujol (registered as B9900005) and approved by the Català government according to current national and European Union legislation on the protection of experimental animals. Mice were supervised daily according to a strict protocol to ensure their welfare and were euthanized, if required, with isoflurane (inhalation excess). Briefly, 50 to 250 million purified CD4$^+$ T cells were infused in 5 mice (10 to 50 million per mouse). Whole blood samples were collected every 2 weeks until week 12, when possible. Plasma was used for quantification of HIV-1 RNA using the m2000 Abbott platform. Whole blood was stained to define human T-cell engraftment as the proportion of human CD45$^+$ cells in the total lymphocyte gate and activated CD4$^+$ T cells (hCD45$^+$CD3$^+$CD8$^-$HLADR$^+$CD69$^+$CD25$^+$) (Fig. 42). We also lysed blood cells and quantified HIV-1 DNA as previously described in chapter 3a. Spleen samples were collected at the last time point and were mechanically disaggregated and used to quantify HIV-1 DNA with droplet digital PCR after lysis of erythrocytes.
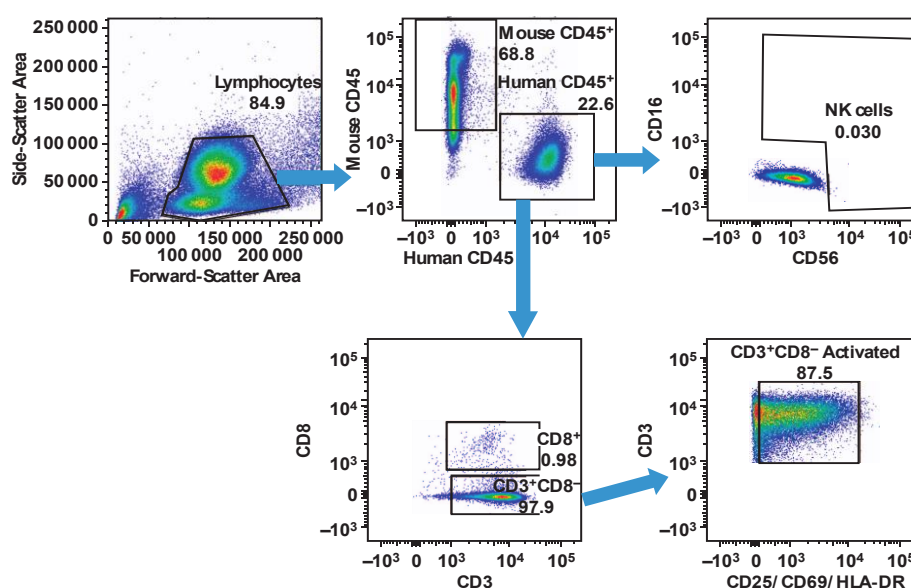
Figure 42. Gating strategy to quantify engraftment of human cells (proportion of human CD45$^+$ cells), CD4 (defined as CD3$^+$CD8$^-$) cell activation, and the event of any CD8 or NK contamination. NK= Natural Killer.

3d.3. RESULTS

3d.3.1. Clinical characteristics of the IciStem cohort individuals

Thirty individuals were transplanted and studied within the IciStem Cohort. 10/30 were transplanted with a *CCR5* mutated donor in homozygosis, 1/10 with a heterozygous *CCR5* donor and the others with a *CCR5* wt donor. The hematological diseases in the cohort were Acute Myeloid Leukemia (9), Hodgkin lymphoma (4), Diffuse large B-cell lymphoma (DLBCL) (4), Burkitt NHL (2), Myelodysplastic Syndrome (2), Plasmablastic lymphoma (2), and others in low proportion (7: Chronic Myeloid Leukemia, Hemophagocytic lymphohistiocytosis, Myelofibrosis, Myeloid Sarcoma, NK-NHL, Non Hodgkin lymphoma and osteomyelofibrosis). 14/30 individuals died after allo-HSCT, 71% of them during first 4 months.

3d.3.2. HIV-1 reservoir decay after allo-HSCT

A reservoir analysis, including total HIV-1 DNA in PBMCs, qVOA and usVL was performed in the 30 individuals that compose the IciStem cohort. It included more than 100 blood samples from different time points before and after allo-HSCT. We observed that the reservoir measured by the three techniques was regularly detectable in baseline samples, but after the allo-HSCT a drastic reduction occurred in the first 1-6 months (Fig. 43a-c). After 6 months, only two individuals remained detectable for usVL (IciS-01 and IciS-20).

Moreover, we did not observe a faster decay in the HIV-1 reservoirs in the individuals that received *CCR5* Δ32/Δ32 cells such as IciS-19, IciS-33, or IciS-36. Therefore, we confirmed that the allo-HSCT by itself can reduce the viral reservoir regardless of the *CCR5* genotype of the donor. Thus, in the following sections we decided to focus in six individuals that received an allo-HSCT with a *CCR5* wild-type donor to study the specific mechanisms that contributed to the decline in viral reservoirs despite not having received *CCR5* Δ32/Δ32 cells that is already known to be a key factor to impede HIV-1 infection.
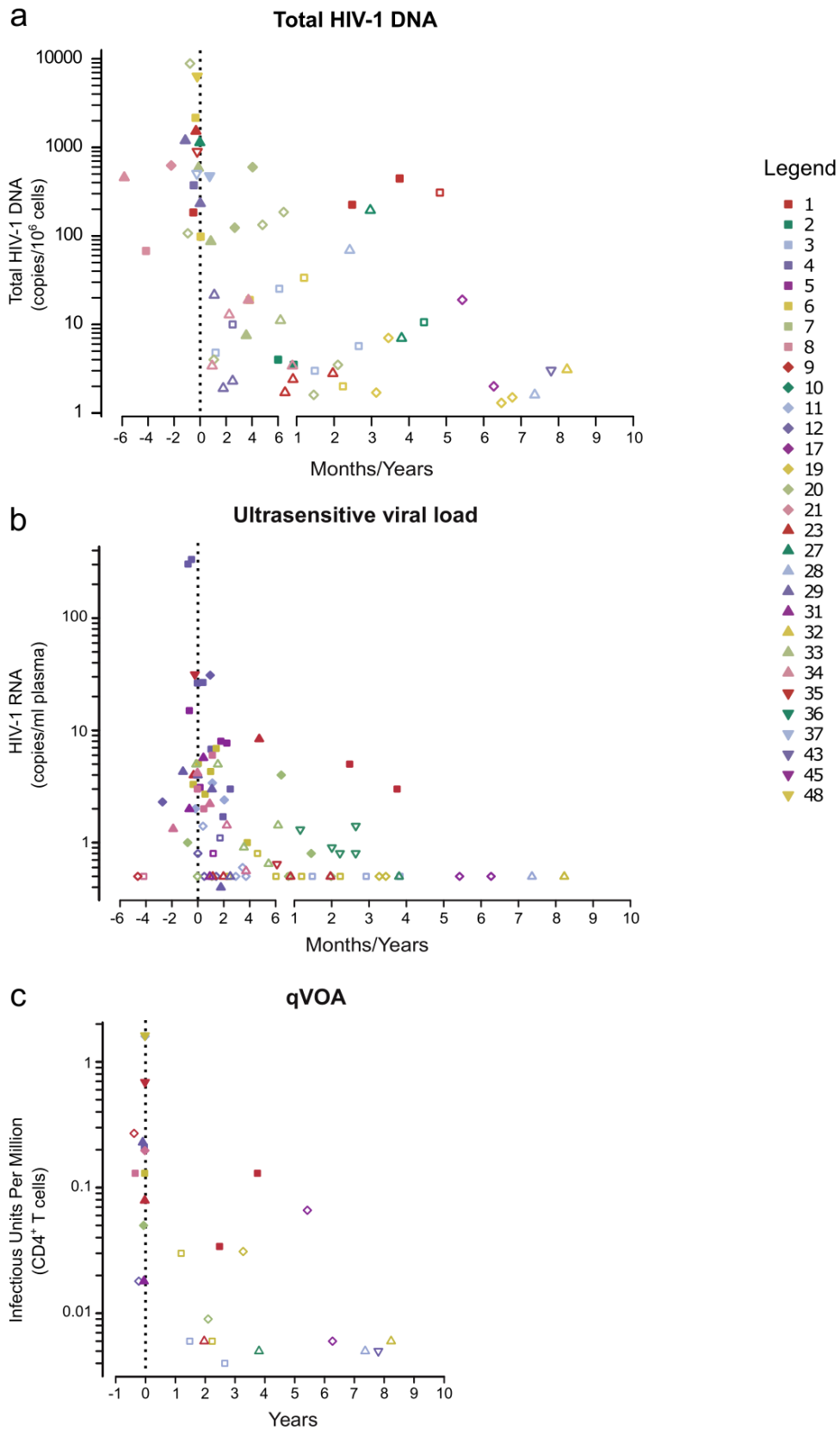
Figure 43. HIV-1 reservoirs measured in blood in the IciStem cohort. (**a**) Total HIV-1 DNA measured in PBMCs by ddPCR, (**b**) ultrasensitive viral load in plasma and (**c**) quantitative viral outgrowth assay measured in CD4+ T cells.

3d.3.3. Clinical characteristics of the long-term transplanted IciStem individuals

We selected six individuals that survived more than two years after transplant with *CCR5* wild-type donor cells, all of them showed complete remission of their hematologic disease, no longer had immunosuppression, and maintained cART during and after transplant; only participant IciS-06 interrupted cART from days 5 to 24 due to severe mucositis, with no evidence of viral rebound. Hematologic and virological characteristics of the individuals are shown in table 9.

Different transplant strategies were used according to the decisions of the participants' hematologists. IciS-01 received a myeloablative single cord blood transplant supported with third-party HLA-mismatched CD34^{+} cells (haplo-cord HSCT)[327,328]. IciS-03, IciS-17, and IciS-27 underwent reduced-intensity, conditioned allo-HSCT from HLA-matched related donors. IciS-06 received a reduced-intensity, conditioned, nonmanipulated transplant from an HLA-haploidentical donor, with posttransplant cyclophosphamide for graft-versus host disease (GvHD) prophylaxis[329]. Finally, IciS-28 received a reduced-intensity, conditioned, HLA-matched transplant from an unrelated donor.

Table 9. Clinical, Hematologic, and Virological Characteristics of the 6 individuals

Characteristic	IciS-01	IciS-03	IciS-06	IciS-17	IciS-27	IciS-28
Clinical characteristics						
Sex	Male	Male	Male	Male	Male	Male
Age, *y*	34	51	40	46	47	44
Country of origin	Spain	Spain	Spain	Italy	Spain	Spain
Hematologic characteristics						
Diagnosis	Burkitt NHL (stage IV)	NK-NHL (stage IV)	HL (stage IV)	NHL (DLBCL)	NHL (stage III)	HL (stage III)
Year of allo-HSCT	2012	2013	2014	2010	2013	2009
Status at transplant	CR2	CR1	CR1	CR2	CR1	CR3
Donor type/graft source	Cord blood 7/8 (mismatch in DRB1) + mismatched related PBPC†	HLA-identical sibling/PBPC	HLA-haploidentical sibling/PBPC	HLA-identical sibling/ PBPC	HLA-identical sibling/PBPC	HLA-identical unrelated/ PBPC
Donor *CCR5* type	*CCR5* wt/wt	*CCR5* wt/wt	*CCR5* wt/wt	*CCR5* wt/wt	*CCR5* wt/wt	*CCR5* wt/wt
Recipient HLA	A*02/02 B*44/51 Cw02/05 DRB1*04/07 DQB1*03/03	A*25/01 B*18/15 Cw12/03 DRB1*13/03 DBQ1*06/02	A*02/03 B*44/51 Cw05/07 DRB1*07/04 DQB1*02/03	A*03/24 B*18/51 Cw12/14 DRB1*07/11 DQB1*02/03	A*01/34 B*08/18 C*07/12 DRB1*01/11 DQB1*03/05	A*26/29 B*44/49 C*07/16 DRB1*01/07 DQB1*02/05
Donor–recipient HLA match	5/6 (DRB1)‡	10/10	6/8 (B*44/57 and DRB1-07/07)	10/10	10/10	10/10
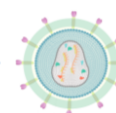
Table 9. Continued

Characteristic	Icis-01	Icis-03	Icis-06	Icis-17	Icis-27	Icis-28
Conditioning	MAC: FLU, CY, busulfan, and ATG	RIC: FLU and melphalan	RIC: FLU, CY, and busulfan	RIC: Thiotepa, FLU, and CY	RIC: FLU and CY	RIC: FLU and melphalan
Transplant-associated infections	*Escherichia coli,* BK virus hemorrhagic cystitis	None	*Clostridium difficile* CMV reactivation	EBV reactivation (treated with rituximab)	None	CMV reactivation
GvHD prophylaxis	CsA + steroids	CsA + Mtx	Post-transplant CY+CsA+ MMF	CsA + Mtx	CsA + Mtx	Tacrolimus + sirolimus
GvHD	No	Chronic: Mild (by month 8), affecting skin	Acute: Severe (by month 3), affecting skin and intestines	No	Chronic: Mild (by month 4)	Acute (by day 12, grade II): affecting skin and intestines Chronic: moderate
Day of neutrophil engraftment	15	18	22	15	11	11
Day of platelet engraftment	31	9	25	16	11	21
Time to complete chimerism, mo§						
Peripheral blood	2	1	3	1	ND	ND
T lymphocytes	18	1	3	ND	5.5	1
Bone marrow	12	6.5	6	ND	ND	ND
Immunosuppression at last follow-up	No	No	No	No	No	No
Status at last follow up	Alive with CR	Alive with CR	Alive with CR	Alive with CR	Alive with CR	Alive with CR
Virological characteristics						
Time from HIV-1 diagnosis to allo-HSCT, *y*	1	27	2	16	8	11
Time from HIV-1 diagnosis to allo-HSCT, *y*	1	27	2	16	8	11
Time from start of cART to allo-HSCT, *y*	1	19	2	13	8	11
HIV-1 tropism	R5	Dual R5/X4	Dual R5/X4	ND	ND	ND
Posttransplant HIV-1 cART	ABC+3TC+ RAL, maraviroc	TDF, FTC, RAL	TDF, FTC, RAL	TDF-FTC+DRV/r +RAL	1.FTC+TDF + EFV 2.ABC+3TC + EFV 3.ABC+3TC + rilpivirine	1.FTC+TDF+ RAL 2.ABC+3TC+ RAL 3.ABC+3TC+ DTV
Plasma VL before allo-HSCT, HIV-1 RNA copies/ml	65	<50	<50	<40	<1	<1
CD4 T-cell count before allo-HSCT, × 10^9 cells/l	0.720	0.800	0.151	0.155	0.747	0.891
CD4 cell count 3 mo after allo-HSCT, × 10^9 cells/l	0.410	0.558	0.324	0.320	0.160	0.390
Maximum CD4 T cell count after allo-HSCT, × 10^9 cells/l	0.891	0.660	0.759	0.773	0.815	2.550
Detectable plasma VL after allo-HSCT	Yes‖	No	No	No	No	No

3TC=lamivudine; ABC=abacavir; allo-HSCT=allogeneic hematopoietic stem cell transplant; ART=antiretroviral therapy; ATG=antithymocyte globulin; CMV=cytomegalovirus; CR=complete remission; CsA=cyclosporine A; CY=cyclophosphamide; DLBCL=diffuse large B-cell lymphoma; DRV/r=darunavir + ritonavir; DTV=dolutegravir;

EBV=Epstein–Barr virus; EFV=efavirenz; FLU=fludarabine; FTC =emtricitabine; GvHD=graft-versus-host disease; HL=Hodgkin lymphoma; MAC=myeloablative conditioning; MMF=mycophenolate mofetil; Mtx=methotrexate; ND=no data; NHL=non-Hodgkin lymphoma; NK=natural killer; PBPC=peripheral blood progenitor cell; RAL=raltegravir; RIC=reduced-intensity conditioning; TDF=tenofovir disoproxil fumarate; VL=viral load; wt=wild-type. † Single cord blood transplant supported with third-party HLA-mismatched CD34+ cells (haplo-cord transplant); ‡ 3/10 with a third-party donor; §Conventional chimerism (polymerase chain reaction of short tandem repeats); ‖ Ultrasensitive VL with limit of detection of 0.5 copy/ml.

All participants achieved complete standard chimerism in peripheral blood and BM in the first 12 months after allo-HSCT (Table 9). IciS-01 showed delayed achievement of complete T-lymphocyte chimerism (18 months) compared with individuals with available data. Data on ultrasensitive chimerism in peripheral blood were available for IciS-01, IciS-03, and IciS-06; only IciS-01 showed mixed chimerism at the last follow-up. Four individuals had posttransplant GvHD; three of them had acute GvHD, and two had chronic GvHD that was treated with immunosuppression (Table 9).

3d.3.4. HIV-1 reservoir characterization of the long-term transplanted IciStem individuals

Comprehensive virological studies were performed in blood and tissue samples from the six participants (Fig. 44 and Table 10). The blood HIV-1 reservoir (proviral HIV-1 DNA analysis and qVOA in blood cells and HIV-1 RNA analysis in plasma) was undetectable in 5 of 6 participants at the last follow-up. Of note, cell input for both HIV-1 DNA analysis and qVOA was similar to that in previous reports of allo-HSCT and substantially higher than in other studies[230,231]. Conversely, low virus levels were consistently detected in blood samples from IciS-01 (453 HIV-1 DNA copies/10^6 CD4+ T cells, 3 HIV-1 RNA copies/ml of plasma, and 0.13 IUPM).

HIV-1 was also undetectable in CSF and cells from BM, LN, and ileal biopsy specimens in all participants, in line with previous observations in blood (Fig. 44 and Table 10).
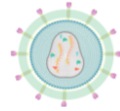
Table 10. HIV-1 latent reservoir in all the samples isolated from each individual in blood and tissues.

Variable	Blood						Tissues							
	qVOA in CD4+ cells		HIV-1 DNA in CD4+ cells		Ultrasensitive VL		Ileum: HIV-DNA in CD45+ cells		Lymph node: HIV-1 DNA in T_{FH} cells		Bone Marrow: HIV-1 DNA in CD4+ cells		CSF: Ultrasensitive VL	
	Cell Input	IUPM	Cell Input	Copies/10^6 cells	Plasma	HIV-1 RNA copies/ml	Cell Input	Copies/10^6 cells	Cell Input	Copies/10^6 cells	Cell Input	Copies/10^6 cells	CSF	HIV-1 RNA copies/ml
IciS-01														
Before SCT	PBMC		PBMC	184										
Month 29	88×10^6	0.034	1×10^6	225	9ml	5							--	--
Month 45	63×10^6	0.129	1×10^6	453	9ml	3							--	--
IciS-03														
Month 17	113×10^6	Neg (<0.006)	1×10^6	Neg (<5)	9ml	Neg (<0.5)	1.5×10^4 (CD4+)	Neg (<64)	--	--	2×10^6 (CD3+)	Neg (<0.5)	2.5 ml	Neg (<0.4)
Month 31	112×10^6	Neg (<0.004)	1×10^6	Neg (<6)	9ml	Neg (<0.5)	1×10^4	Neg (<98)	--	--	3.7×10^7	Neg (<0.03)	3ml	Neg (<0.3)
IciS-06														
Before SCT	63×10^6	0.130	1×10^6	2162	9ml	3	1.4×10^4 (CD4+)	4000	--	--	--	--	--	--
Month 15	23×10^6	Neg (<0.031)	1×10^6	Neg (<9)	9ml	Neg (<0.5)	3×10^4	Neg (<34)	3×10^3	Neg (<342)	8×10^5	Neg (<1)	3ml	Neg (<0.3)
Month 27	113×10^6	Neg (<0.006)	1×10^6	Neg (<2)	9ml	Neg (<0.5)	5.4×10^3	Neg (<185)	4.5×10^3	Neg (<222)	5.5×10^4	Neg (<18)	3ml	Neg (<0.3)
IciS-17														
Month 65	11×10^6	Neg (<0.031)	1×10^6	Neg (<19)	9ml	Neg (<0.5)	--	--	--	--	--	--	--	--
Month 75	112×10^6	Neg (<0.006)	1×10^6	Neg (<2)	9ml	Neg (<0.5)	2×10^5	Neg (<5)	6.3×10^4 Bulk LN	Neg (<16)	6.3×10^5	Neg (<2)	7ml	Neg (<0.1)
IciS-27														
Before SCT	PBMC		PBMC	1137										
Month 45	137×10^6	Neg (<0.005)	1×10^6	Neg (<7)	9ml	Neg (<0.5)	3×10^4	Neg (<33)	--	--	3×10^5	Neg (<3)	3ml	Neg (<0.3)
IciS-28														
Month 88	137×10^6	Neg (<0.005)	1×10^6	Neg (<8)	9ml	Neg (<0.5)	2.7×10^5	Neg (<4)	2.3×10^3 (CD4+)	Neg (<435)	2.7×10^5	Neg (<4)	3ml	Neg (<0.3)

Limit of detection is shown for negative values. Neg= negative; CSF= cerebrospinal fluid; qVOA= quantitative viral outgrowth assay; IUPM= infectious units per million cells; LN= lymph node; PBMC: peripheral blood mononuclear cells; T_{FH}: T-follicular helper cells; Neg: negative; VL= viral load.
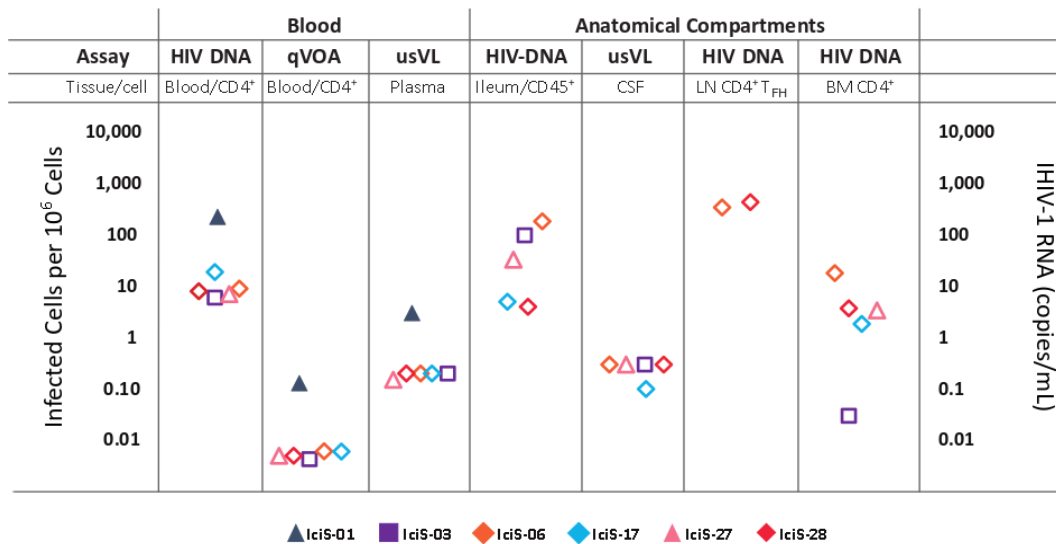
	Blood			Anatomical Compartments				
Assay	HIV DNA	qVOA	usVL	HIV-DNA	usVL	HIV DNA	HIV DNA	
Tissue/cell	Blood/CD4+	Blood/CD4+	Plasma	Ileum/CD45+	CSF	LN CD4+ T$_{FH}$	BM CD4+	

Figure 44. HIV-1 Reservoirs measured in blood and tissues after transplant. Data are from the last collected sample for each individual. Open symbols represent undetectable values (only IciS-01 had detectable values). In those cases, the limit of detection for the samples varied based on cell/volume input, and that value is represented. BM= Bone marrow; CSF= cerebrospinal fluid; qVOA= quantitative viral outgrowth assay; LN= Lymph node; usVL= ultrasensitive Viral Load; T$_{FH}$= T-follicular helper cells.

3d.3.5. Longitudinal correlation of hematologic and HIV-1 reservoir parameters

Five of six participants had undetectable HIV-1 reservoirs (Fig. 45). All five had peripheral blood progenitor cells as the graft source; for developed GvHD; and all five achieved complete chimerism in peripheral blood, BM, or T lymphocytes within the first year after transplant. Conversely, the only participant with a detectable HIV-1 reservoir (IciS-01) received a cord blood transplant with a conditioning regimen that contained antithymocyte globulin (ATG). This participant did not develop GvHD and had mixed chimera in T cells up to posttransplant month 18, as measured by standard methods.

Patient	HIV Reservoir	Stem Cell Donor	Time to Full Donor Chimera in T Lymphocytes	Graft-Versus-Host Disease
IciS-01	Detectable	Cord blood	>1 y	None
IciS-03	Undetectable	PBPC	<1 y*	Acute
IciS-06	Undetectable	PBPC	<1 y	Acute
IciS-17	Undetectable	PBPC	ND	None
IciS-27	Undetectable	PBPC	<1 y	Chronic
IciS-28	Undetectable	PBPC	<1 y*	Acute and Chronic

Figure 45. Relationship between latency parameters measured in each participant and clinical

conditions of each allogeneic stem cell transplant. ND=not determined; PBPC= peripheral blood progenitors. *Full donor chimera within a month.

Longitudinal follow-up of IciS-06 is shown in Fig. 46. This participant showed mixed chimerism in peripheral blood in the first few weeks after transplant, with concomitant detection of persistent reservoirs. By month 3, the individual developed acute grade III GvHD after withdrawal of immunosuppression coinciding with achievement of full donor chimerism. Coincidentally, residual viremia became undetectable in plasma. Cell associated HIV-1 DNA was also undetectable at that point, showing a 2-fold reduction in just 4 months. Clinical data suggest that similar phenomena may have occurred in the other four individuals given that all had full chimerism within 6 months after allo-HSCT and/or GvHD (Table 9).
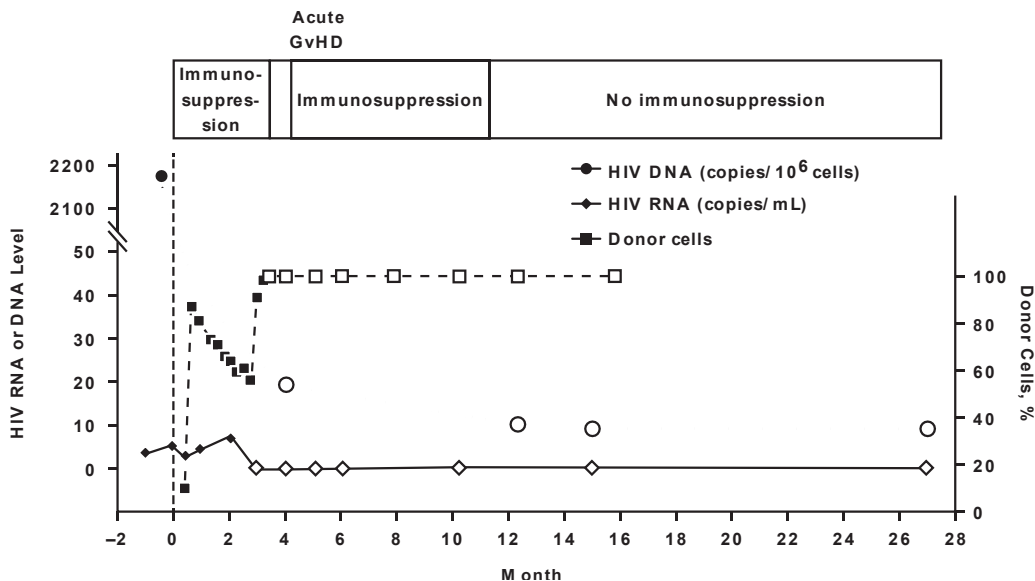
Figure 46 Peripheral blood standard donor chimerism, proviral HIV-1 DNA, and plasma HIV-1 RNA evolution after transplant in IciS-06. Open diamonds and circles indicate undetectable HIV-1 RNA (ultrasensitive viral load) and proviral HIV-1 DNA, respectively, and represent the limit of detection of each technique, which is based on cell/plasma input. For chimerism expressed as percentage of donors, open squares indicate full donor chimera. GvHD= graft-versus-host disease.

3d.3.6. HIV-1 specific humoral response

We explored HIV-1 humoral response dynamics in plasma samples after allo-HSCT (Fig. 47). All participants lost the p18 band. We observed no other missing bands in IciS-01; however, IciS-03 and IciS-06 showed decreasing p31 antibody levels, and IciS-06 and IciS-17 lacked p55 and p24 bands. More important, we did not detect any viral antibodies in IciS-28 by month 88, suggesting that this individual experienced seroreversion. Overall, a longer interval after allo-HSCT seemed to be

associated with greater antibody clearance among individuals receiving cART. These data were confirmed with the low sensitivity VITROS analysis, which showed decreased levels of HIV-1 antibodies and a progressive loss over time after allo-HSCT (Fig. 48). IciS-28 also showed antibody levels close to those of the HIV-negative donors. Overall, the data suggest limited de novo humoral responses that could sustain the HIV-1 specific immunoglobulin levels in the plasma of these individuals.
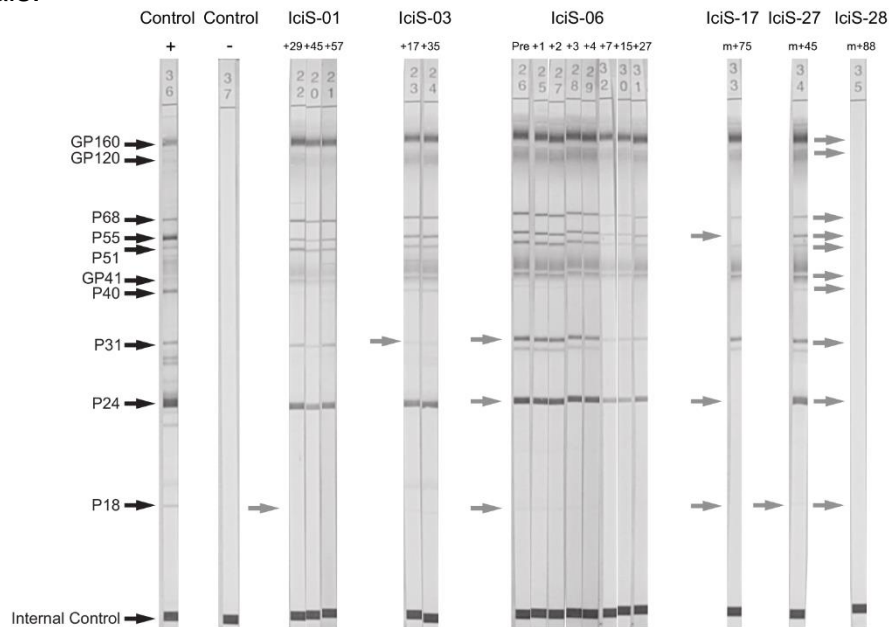
Figure 47. Western blot analysis from the six analyzed individuals. Grey arrows indicate bands left or reduced intensity in each case.

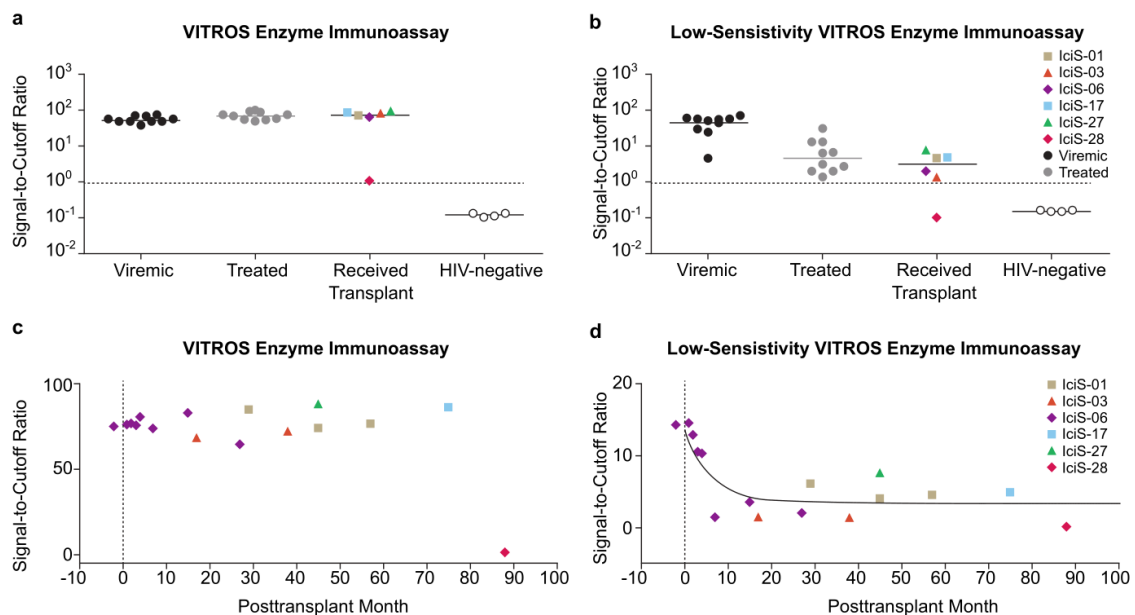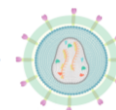**Figure 48. HIV-**1 specific antibody determination using the VITROS enzyme immunoassay. (**a**) Absolute antibody quantification in the last sample from each transplant recipient compared with viremic and treated HIV-1 positive individuals and HIV-negative donors. (**b**) Detuned low-sensitivity

antibody quantification in the last sample from each transplant recipient compared with viremic and treated HIV-1 positive individuals and HIV-negative donors. (**c**) Absolute antibody quantification in longitudinal plasma samples from all included individuals. (**d**) Detuned low-sensitivity antibody quantification in all included individuals

3d.3.7. Humanized mouse VOA

We transferred large numbers of CD4$^+$ T cells purified from the individuals' peripheral blood to immunosuppressed mice to detect any replication-competent blood cell reservoir (Fig. 49a). As a control, we also transferred cells from an HIV-1-infected individual who had not undergone transplant, was receiving long-term cART, and had a standard HIV-1 reservoir size (1.6 IUPM)[166].
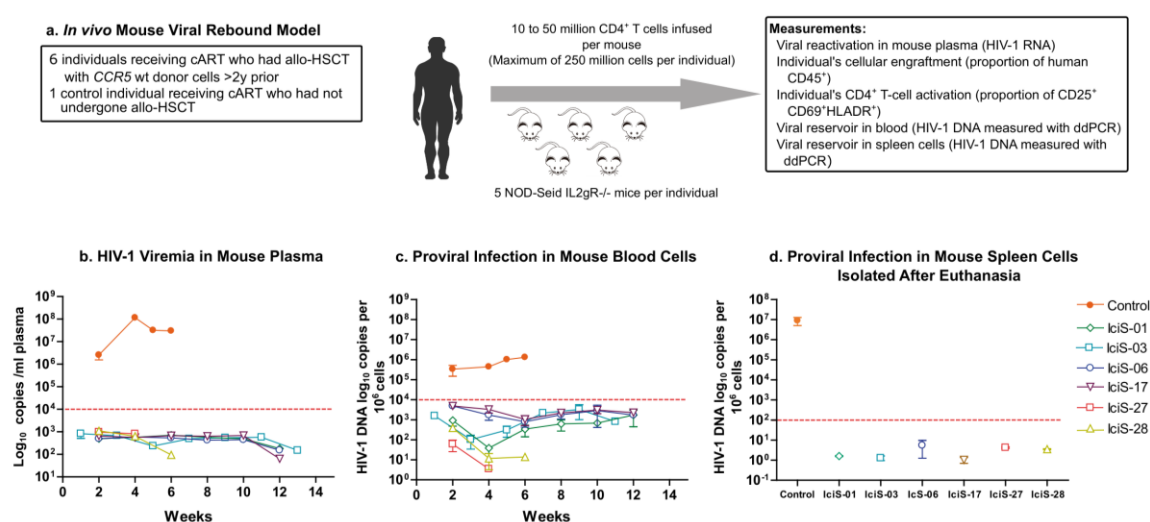
Figure 49. In vivo mouse viral rebound model. (**a**) Model and sample approach. (**b**) Quantification of viremia in mouse plasma. (**c**) Proviral infection in mouse blood cells. (**d**) Proviral infection in mouse spleen cells isolated after euthanasia. All six individuals had undetectable values. Open symbols represent undetectable values. Limit of detection relative to plasma volume input is shown. Error bars represent medians and interquartile ranges of the values from the five mice used for each individual. allo-HSCT = allogeneic hematopoietic stem cell transplant; cART= combination antiretroviral therapy; ddPCR= droplet digital polymerase chain reaction; wt= wild-type.

We detected high levels of HIV-1 RNA in the plasma of humanized mice infused with control CD4$^+$ T cells (Fig. 49b). Cell-associated HIV-1 DNA was also detected in blood and spleen cells from the same infected mice (Fig. 49c-d). Conversely, none of the mice infused with cells from the six allo-HSCT recipients had detectable virus in plasma or cell-associated HIV-1 DNA in the blood or spleen after 4 to 13 weeks of follow-up. Of note, median survival of the mice was 6 weeks [IQR: 5 to 12 weeks]. Also, the median of maximum engraftment of human lymphocytes in the mice was 34% [15% to 45%]. Among engrafted human CD4$^+$ T cells, activation levels reached

a median of 95% [80% to 97%], suggesting optimal conditions for eventual HIV-1 reactivation.

Because IciS-01 did not show reactivation, we also tested cells from an HIV-1-infected individual who did not undergo transplant, was receiving cART, and had a similarly small HIV-1 reservoir (0.13 IUPM). HIV-1 DNA (1000 copies/10^6 cells) was detected in the spleen and blood of mice with human cells transferred from this person, proving the robustness of the technique. This model suggested that immediate viral rebound was not likely after discontinuation of cART in the six long-term transplanted recipients. Moreover, the virus in IciS-01 might have low inducibility under in vivo physiologic conditions.

3d.3.8. Viral rebound after cART interruption in one individual

IciS-28, after suffering a psychiatric condition, decided voluntarily to interrupt cART in September 2018, without medical supervision. In January 2019, 4 months after, IciS-28 reported symptoms compatible to an acute HIV-1 syndrome, and a viral load of 100,000 copies/ml was detected (Fig. 50). The individual reported no risk of superinfection during the period without cART. Sequence comparison was not possible since no virus could be amplified before treatment interruption. In February 2019 (month +113 posttransplant) the participant resumed cART.
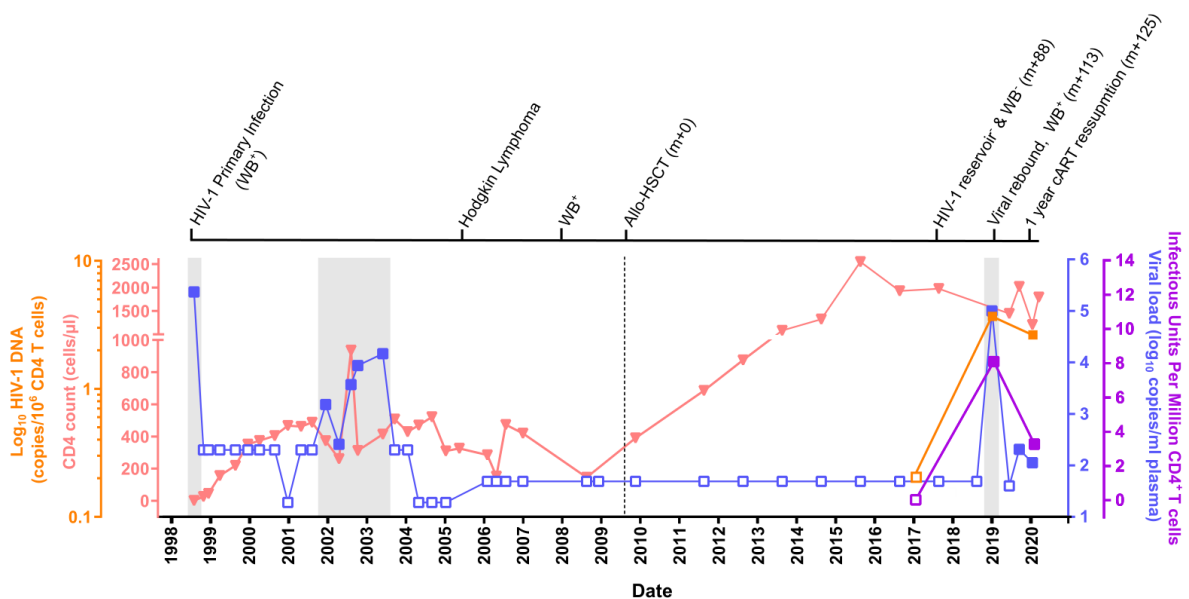
Figure 50. Clinical course of IciS-28 before and after allo-HSCT. CD4 counts, plasma viral load total HIV-1 DNA and infectious units per million are shown over time. Grey shaded areas indicate periods without cART. Open symbols represent undetectable values, where the limit of detection is represented based on cell/volume input. WB= western blot.

An analysis of their viral reservoir was performed before cART interruption (m+88), at viral load rebound (m+113) and after 1 year of cART resumption (m+125). As previously shown, at m+88 Icis-28 had a viral reservoir below 0.005 IUPM. However, once cART was interrupted, we were able to detect replication competent cells with an IUPM of 8.066. Unfortunately, although the resumption of cART decreased the levels of replication-competent cells, it did not reach the undetectable levels present before the interruption of cART. The same occurred with the levels of total HIV-1 DNA.

Moreover, the negative western blot observed at m+88 became positive once the viral load rebounded at m+113 (Fig. 51)
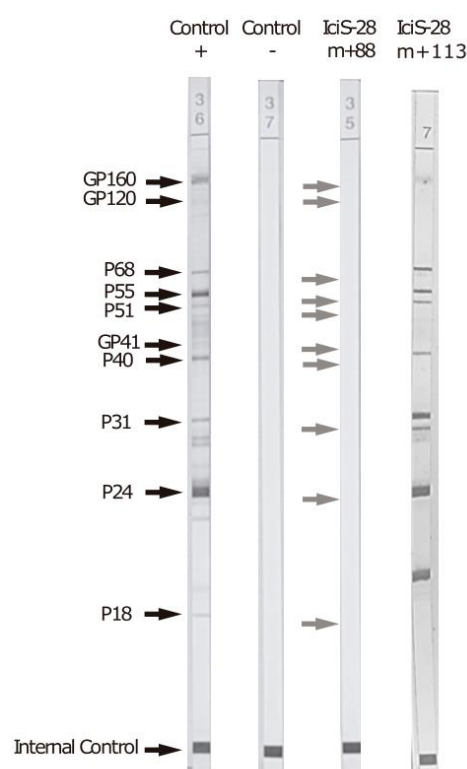
Figure 51. Western blot analysis from the Icis-28 before (m+88) and after treatment interruption (m+113). Grey arrows indicate bands left or reduced intensity in each case.

This is a proof of concept that even with an extreme reduction of the HIV-1 reservoir, as observed in this individual, an additional reactivation inhibition mechanism (e.g. *CCR5* mutation, immune response, etc) seems to be necessary to achieve an HIV-1 cure.

3d.4. DISCUSSION

Previous studies have shown that allo-HSCT can result in a significant reduction in the latent HIV-1 reservoir[231,322–324] and, in unique cases linked to transplant of *CCR5*-mutated cells, even eradication of the virus[229,230,233,234], making HIV-1 cure a feasible target. However, the specific mechanisms that contributed to the decline in viral reservoirs in these individuals are not fully understood, in part due to scant experience with allo-HSCT in HIV-1-infected individuals. The IciStem consortium provides an opportunity to exhaustively study HIV-1 remission in multiple HIV-1-infected individuals who have undergone allo-HSCT, including the six long-term survivors described in detailed in this article. Not only have we confirmed the reduction of the HIV-1 reservoir in blood and plasma in 30 individuals from the IciStem cohort[231,322], but five of six participants eliminated any measurable HIV-1 reservoir, as determined by highly sensitive techniques (10 to 100 times more sensitive than those used in previous studies[167]) in LNs, ilea, BM, and CSF.

The only individual who had a detectable reservoir underwent cord blood allo-HSCT with an ATG containing conditioning regimen, did not develop GvHD, and had longer persistence of recipient cells in the T cell compartment. All the other participants, who did not have a detectable reservoir, reached full donor chimerism within a year, and four of them developed GvHD, although we cannot confirm that those events converged in time for all of them. Exhaustive follow-up of one of the participants with complete viral clearance showed that HIV-1 became undetectable coincidentally with achievement of complete donor chimerism and development of GvHD.

These results are in line with those of previous reports, where episodes of GvHD and achievement of complete chimerism also coincided with substantial reductions in the viral reservoir[229,231,322,324]. In contrast to the Boston patients, the IciStem participants included in our study were all free of immunosuppression at the last follow-up with T-cell immune reconstitution and had longer posttransplant survival. HIV-1 specific seroreversion at 8 years after transplant in IciS-28 suggests that longer time to remission might contribute to HIV-1 clearance.
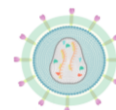
We postulate that replacement of recipient hematopoietic cells with donor cells (that is, achievement of complete chimerism in all compartments, with subsequent exertion of alloreactivity by the healthy donor immune system) might be a major factor in HIV-1 remission after wild-type donor transplantation, as previously suggested[231]. After allo-HSCT, graft-versus-host immune responses against allelic variants of major and minor histocompatibility complex molecules contribute to a graft-versus-leukemia effect that is the basis of the therapeutic effect of allo-HSCT on hematologic disease[330]. Similarly, this potent alloreactive immune effect may contribute to the reduction of latently HIV-1-infected recipient cells through a "graft-versus-HIV-reservoir" effect.

Specific transplant-associated characteristics may explain why HIV-1 persisted in IciS-01. For example, the immunosuppressive effect of ATG combined with use of a less mature graft source (cord blood cells) may have moderated the potential graft-versus-HIV-reservoir effect. Delayed immune T-cell reconstitution is the primary drawback of cord blood transplants because of the immature nature of the engrafted cells. *In vivo* profound T-cell depletion resulting from ATG-containing regimens further intensifies long-lasting impairment of immune reconstitution. Better T-cell recovery with lower incidence of virus reactivation and death from viral infection has been reported in cord blood transplant recipients not receiving ATG[331].

On the other hand, whether the absence of clinically significant GvHD in this setting also played a role is difficult to determine with certainty. Clinically evident GvHD is one of the manifestations of alloreactivity when graft-versus-host immune responses target recipient tissues other than hematopoietic cells, and it occurs frequently after allo-HSCT. Of note, the ability of cord blood cells to exert potent antitumor activity has also been observed with low rates of GvHD[332]. However, the long-term persistence of recipient cells in the T-cell compartment in IciS-01 clearly contrasts with the achievement of complete chimerism in the other participants. Evaluation of additional persons without GvHD is needed to better understand the role of GvHD among the other potential factors associated with allogeneic transplant in the eradication of HIV-1.

Finally, the specific posttransplant immunosuppressive regimen may also play a role in this setting. After infusion of donor hematopoietic cells, posttransplant immunosuppression exerts its inhibitory effects mainly in donor immune cells to prevent severe GvHD. This could have a dual effect in the setting of HIV-1 infection depending on the mechanism of action, dose, and timing. On one hand, it could prevent or modulate alloreactivity against the residual HIV-1 reservoir; on the other hand, it could also limit uninfected T-cell permissiveness for HIV-1 replication, thus maximizing reservoir reduction. Posttransplant high-dose cyclophosphamide (participant IciS-06), which is commonly used for GvHD transplantation, eliminates rapidly proliferating alloreactive T cells of both donor and recipient origin and preserves resting memory T cells, which results in effective prevention of GvHD; this represents a potent graft-versus-leukemia effect together with relatively rapid immune reconstitution[333]. Whether this strategy or other classic approaches could also enhance reservoir reduction deserves further investigation. Our series prevents definitive conclusions given the limited number of individuals and their differing GvHD prophylaxis schemes. However, the fact that all participants had discontinued immunosuppressive therapy and five had an undetectable HIV-1 reservoir at their last assessment suggests a positive effect of immunosuppression withdrawal in preserving alloreactivity against both the underlying hematologic disease and the HIV-1 reservoir. The independent contribution of these factors is difficult to evaluate in the present study, but the results suggest a multifactorial interaction that promotes HIV-1 remission.

We used an *in vivo* humanized mouse model that has proved highly sensitive in detecting replication competent reservoir in HIV-1 ECs[168]. Infusion of CD4$^+$ T cells from an HIV-1-infected control individual who had not undergone transplant led to virus reactivation in the plasma within 2 weeks, similar to what has been described elsewhere[334]. In contrast, virus reactivation was not observed in mice infused with cells from participants who underwent allo-HSCT. This included IciS-01, who had shown low but detectable levels of replication-competent virus when the ultrasensitive qVOA technique was used. The unusually high numbers of CD4$^+$ T cells included in our qVOA[168,335] with strong phytohemagglutinin-mediated stimulation enhanced virus reactivation in these cultures over physiologic conditions
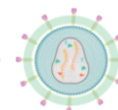
in mice. It seems reasonable that in the absence of cART, IciS-01 would have a longer HIV-1 reactivation period than expected because this participant harbored a small replication-competent reservoir. However, we cannot rule out later HIV-1 rebound after interruption of cART for any of the participants with undetectable reservoir levels, as happened in previous reported cases[231,322–324].

In fact, after a voluntarily treatment interruption IciS-28 suffered from an abrupt viral rebound, indicating that undetectable viral reservoirs and HIV-1 seroreversion would not prevent from a viral rebound if the allo-HSCT is performed with a *CCR5* wild-type donor. Therefore, to achieve the HIV-1 remission in the setting of *CCR5* wild-type donor allo-HSCT, it would be required an additional strategy that boost the HIV-1 specific immune responses, such as the use of HIV-1 neutralizing antibodies[222] or immune check point inhibitors[206,207,209,210]. The combination of the profound viral reservoir decay by the allo-HSCT with the boost of the immune system seems to be key to achieve an HIV-1 cure.

Allo-HSCT is indicated for only a small subset of HIV-1-infected persons with underlying hematologic disease because of the high morbidity and mortality associated with the procedure. In those who survive in the long term, exhaustive consecutive studies using highly sensitive techniques could provide important information for better design of efficient, less toxic HIV-1 cure strategies that could apply to the broader HIV-1-infected population.

In conclusion, our study shows that allo-HSCT yielded a profound long-term reduction in the HIV-1 reservoir and HIV-1 specific antibodies in the first 6 months after the transplant, even in the *CCR5* wild-type donor setting. Transplant-associated factors such as the graft vs host disease, an adult donor cell transplant or the time of engraftment have contributed to achieve this reduction. However, the treatment interruption in one individual with a *CCR5* wild-type donor resulted in viral rebound, suggesting that a potent and sustained HIV-1 specific response would be needed in combination to achieve an HIV-1 cure.

CHAPTER 4. DISCUSSION

HIV-1 has caused the death of millions of people since its discovery in the 80's. The development of combination antiretroviral therapy (cART) was one of the major medical success of the 20th century as it effectively suppress plasma viremia to undetectable levels, restores the immune system, reduces mortality and morbidity, and the risk of viral transmission[56,58,336–338]. However, cART is not able to eliminate latently infected cells; therefore, in most individuals when cART is interrupted the latently infected cells can reactivate and establish systemic viral rebound within 2-8 weeks[61].

It is known that the number of latently infected cells is a predictor of disease progression in primary infection[286] and during the natural course of HIV-1 infection[287], and it also predicts time to viral rebound upon cART interruption[295,339]. Therefore, a great variety of strategies are currently focused on eliminating or reducing the latent reservoir to achieve a sterilizing or functional cure with limited success yet[285], besides the allo-HSCT with *CCR5*Δ32 donor cells[229,232–234]. However, some HIV-1-infected individuals can control HIV-1 replication for a certain period of time in the absence of cART without the need of any clinical intervention. These individuals include elite controllers[246] and post-treatment controllers[72], which are characterized by harboring very low levels of latently infected cells, demonstrating the importance of this factor for achieving an HIV-1 cure.

This thesis aimed at improving our understanding of the clinical, immunogenetic and virological factors associated to reductions of the HIV-1 reservoir in three models of HIV-1-infected individuals with extremely low levels of HIV-1 reservoir in order to know the importance of reducing the reservoirs to achieve an HIV-1 cure.

First, in chapter 3a we characterized three individuals with an extreme phenotype of elite controller who were able to control HIV-1 replication for more than 25 years in the absence of cART and whom we called Exceptional Elite Controllers (EEC). Our results showed that EEC were enriched in protective host genetic factors with also highly effective HIV-1 specific immune responses. Both factors were interrelated since the presence of protective HLA alleles seems to be related to better and more potent HIV-1 specific immune responses[340,341]. These two factors, have been previously seen in other studies involving regular elite controllers[247,248].
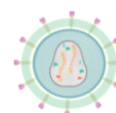
Moreover, we described for the first time an almost absence of viral diversity and evolution of the EEC's provirus, which was probably due to their replicative-impaired and highly defective genomes. Altogether, EEC might be considered as spontaneous cases of HIV-1 functional cure which was probably attained by the combination of **defective non-evolving virus** with a **potent HIV-1 specific immune system** (Fig. 52).
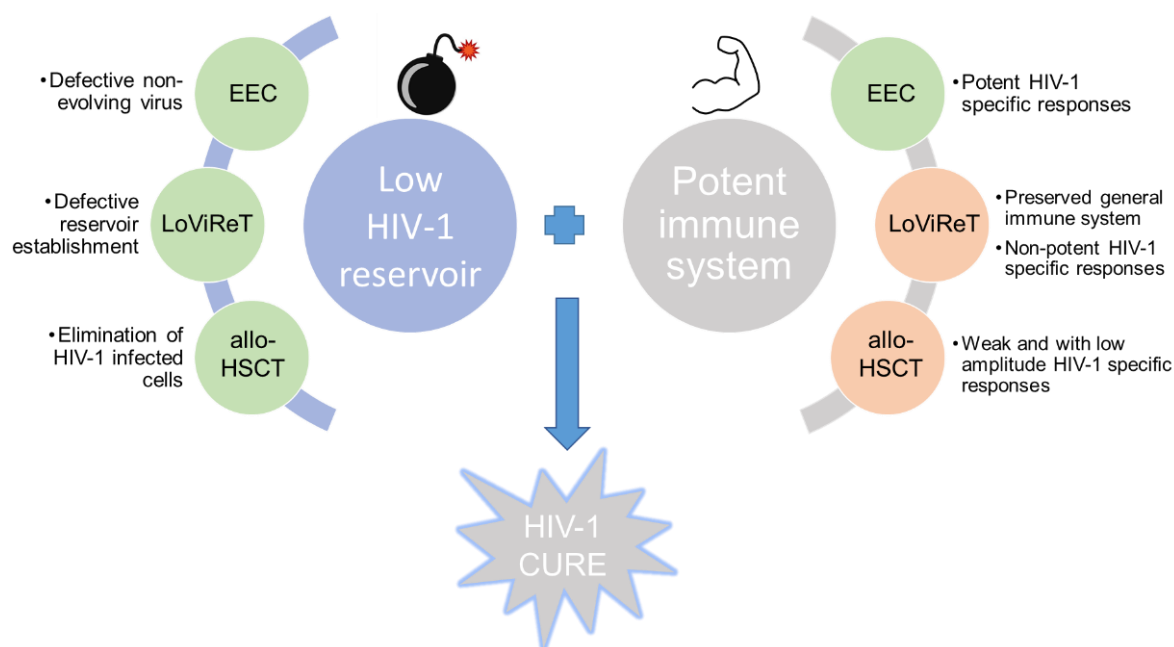
Figure 52. Virological and immunological factors present in the three models of HIV-1-infected individuals that might be necessary to achieve the HIV-1 cure. In green are represented beneficial factors and in red the detrimental ones.

On the other hand, it is known that exist individuals with a very low viral reservoir but that are not able to control HIV-1 replication in the absence of cART[241,242]. Therefore, in chapter 3b we characterize a cohort of LoViReT (Low Viral Reservoir Treated) individuals that have a very low viral reservoir (<50 copies/10^6 PBMCs) and had been under cART for at least three years to control HIV-1 replication. We found that LoViReT accounted for the 9% of all the HIV-1-infected population, with two thirds of them treated in the chronic phase of the infection. The clinical factors associated with being LoViReT were a high CD4 T cell count and lower viral loads before initiation of cART, and a better control of HIV-1 replication under cART. Also, they were characterized by a naturally reduced and more cART-sensitive reservoir, which might indicate a **defective reservoir establishment**. Although they had a better preservation of the general CD8$^+$ T cell compartment, their immune cells

seemed to not being able of control the virus since they had low circulating HIV-1 specific antibodies and their CD8 and NK cells were not able to control the virus *in vitro*, suggesting the **absence of potent HIV-1 specific responses** (Fig. 52).

Furthermore, in chapter 3c we analyzed immunogenetic factors associated with LoViReT and found that they did not have an overrepresentation of protective HLA alleles as happened in EEC, which aligned with the mild HIV-1 specific immune responses observed. When we comprehensively analyzed viral factors, we barely detected replication-competent virus in peripheral blood and found limited provirus in anatomical compartments. Interestingly, we found that the factor that might have contributed to the low non-inducible reservoir and their pronounced decays once they started cART was a skewed distribution of the HIV-1 DNA among the different CD4$^+$ T cells subpopulations. The distribution was shifted towards shorter live cell subpopulations as T_{TM} and T_{EM} that are easier to be eliminated through cART due to their higher composition of unstable viral forms[313], which is in line with what was observed in PTC[72]. Unfortunately, despite their low reservoir levels they are not able to control the virus in the absence of cART probably due to their lack of HIV-1 specific immune responses. Thus, the low reservoir found in LoViReT might be the result of a combination of a defective reservoir establishment before cART, with a faster decay after treatment by the skewed distribution of provirus among the different CD4$^+$ T cells subpopulations.

Finally, in chapter 3d we evaluated the factors associated to the reservoir decay after an allogeneic hematopoietic stem cell transplant (allo-HSCT), which by now is the only cure strategy that have succeeded in this aim[229,232–234]. We confirmed that the allo-HSCT resulted in a profound long-term reduction of the HIV-1 reservoirs, which occurred mainly in the first 6 months after the transplant. An extensive analysis of six long-term followed HIV-1-infected individuals who received an allo-HSCT from *CCR5* wild-type donors, showed that the HIV-1 reduction resulted also in an extremely reduction of the virus in anatomical compartments with a decline of HIV-1 specific antibodies. The factors associated to this reduction included the type of stem cell donor, the time to full donor chimera and graft vs host disease. Those factors generate a "graft-versus-HIV effect" which **eliminates the latently infected cells** from the body that are replaced by the donor non-infected cells (Fig. 52). Also,
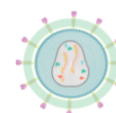
no viral rebound was observed when individual's CD4$^+$ T cells were injected into NOD mice, analyzed by the mVOA assay. Unfortunately, after a voluntarily treatment interruption of one of the individuals an abrupt viral load rebound was observed despite a previous complete seroreversion. This is in line with previous reports with individuals receiving an allo-HSCT with *CCR5* wild-type cells[325].

Overall, these three types of cohorts have in common the extremely low reservoir in peripheral blood and anatomical compartments (analyzed in LoViReT and allo-HSCT individuals) achieved either spontaneously or after an intervention. However, as demonstrated with LoViReT and transplanted individuals, a low reservoir by itself was not sufficient to control the viral replication. One of the different factors that distinguished EEC from LoViReT/allo-HSCT individuals is the potent HIV-1 immune responses they have. In the case of LoViReT, although we observed a preserved general immune system, they have weak HIV-1 specific immune responses. Similarly, allo-HSCT individuals had a strong unspecific response of the immune system mediated by the graft-vs-HIV effect but the HIV-1 specific responses after the allo-HSCT were weak and with low amplitude, as it was recently published by Eberhard *et al*[342].

Thus, the potent HIV-1 specific immune (or alternatively an HIV-1 restriction factor as CCR5 Δ32 mutation) seems to be key to control HIV-1 replication. However, as has been previously shown by different therapeutic interventions, boosting only the HIV-1 specific immune responses is not enough to achieve the HIV-1 cure[213,214]. Therefore, the combination of a low HIV-1 reservoir with potent HIV-1 specific immune responses seems to be crucial to achieve a sustained viral control, as observed in EEC (Fig. 52). In the context of a low reservoir the potent HIV-1 specific immune responses would prevent from viral replication once cART is interrupted.

To overcome the lack of low HIV-1 specific immune responses present in LoViReTs and allo-HSCT individuals, some clinical interventions might be applied. One available approach might be the use of immunotherapies like HIV-1 neutralizing antibodies such as the 3BNC117 (a potent human CD4 binding site antibody) and 10-1074 (targets the V3 glycan supersite on the HIV-1 envelope)[221] which have been

recently demonstrated that boosted the CD8 cytotoxic activity, and in some individuals prevent viral replication when cART is interrupted[222]. Another possibility is the use of monoclonal antibodies against immune check point inhibitors such as PD-1 or PD-L1[206,207,209,210], which have also demonstrated to enhance HIV-1 specific immune responses. Alternatively, gene therapy could be performed to disrupt the *CCR5* gene in autologous CD4$^+$ T cells or hematopoietic stem cells to mimic the natural *CCR5* Δ32 mutation and make the cells resistant to the infection. The use of one of these approaches in combination with the low or even non-detectable reservoirs observed in LoViReT and allo-HSCT may help to achieve a functional or eradication cure.

Additionally, it would be necessary a deeper characterization of the cohort of LoViReT individuals to increase the understanding of the underlying mechanisms and the interplay between host and viral factors that might explain their defects in the reservoir establishment, as well as the skewed distribution among CD4$^+$ T-cell subpopulations. This may include the transcriptomic and genome-wide methylation profile, as well as the genetic and functional integrity of the provirus sequences. The information obtained might help to design new strategies focused on achieving the low reservoir factor necessary to control the HIV-1 replication. Also, it would be interesting to expand the cohort to have a higher number of individuals in which try therapeutic strategies aimed at improving the immune system factor necessary to control the HIV-1 replication.

In summary, attaining a low reservoir seems to be a key factor to achieve a potential HIV-1 cure, but not the only one. Thus, individuals with an already diminished reservoir, naturally or after an intervention, would be closer to achieve an HIV-1 cure. These individuals would be of great interest for trying eradicating strategies aimed at boosting HIV-1 immune specific responses.

CHAPTER 5. CONCLUSIONS

Objective 1: To deeply characterize clinical, virological and immunological factors associated with natural HIV-1 control for more than 25 years in the absence of cART.

1.1. Exceptional Elite Controllers were defined as a subset of HIV-1-infected individuals able to control HIV-1 replication for more than 25 years in the absence of antiretroviral treatment. These individuals can be considered as cases of spontaneous cure.

1.2. Exceptional Elite Controllers harbored an extremely low non-inducible reservoir in blood despite that their CD4$^+$ T cells were susceptible to the infection.

1.3. The main virological factor defining Exceptional Elite Controllers was the presence of highly defective viruses that are non-genetically evolving or diversifying.

1.4. Exceptional Elite Controllers were enriched in protective host factors and had strong cellular HIV-1 immune responses, as previously observed in regular elite controllers.

Objective 2: To determine the clinical, virological and immunological factors related to a reduction of the HIV-1 reservoir in a cohort of LoViReT (Low Viral Reservoir treated) individuals whom harbor an unusual extremely low viral reservoir, but without control of HIV-1 in the absence of cART. Those factors will be related to the longitudinal dynamics of the HIV-1 reservoir and the immune cells before and after initiation of cART between chronic treated LoViReT and controls.

2.1. A total of 9% of the HIV-1-infected individuals under treatment harbored less than 50 HIV-1 DNA copies/10^6 PBMCs, constituting the LoViReT cohort. From them, 66% were treated in the chronic phase of the infection.

2.2. Clinical factors associated to LoViReT were higher CD4 nadir, lower viral loads in the absence of cART, and a better sustained HIV-1 suppression under antiretroviral therapy.

2.3. Intrinsically reduced total HIV-1 DNA and an enhanced decay of reservoirs after initiation of treatment were observed in the cp-LoViReT individuals, suggesting an impaired HIV-1 reservoir establishment.

2.4. A preserved CD8$^+$ T-cell compartment was observed in cp-LoViReT before treatment initiation, but HIV-1 specific responses did not differ from controls.

2.5. Lower HIV-1 specific antibodies on cART were more frequent in cp-LoViReT individuals.

Objective 3: To deeply characterize the host immunogenetic and viral factors associated with the LoViReT cohort, including the replication-competence of the virus, and distribution among anatomical and cellular compartments.

3.1. The LoViReT phenotype were not associated with protective host factors such as an overrepresentation of HLA B-57 or B-27 alleles or *CCR5* Δ32 gene mutation in heterozygosis.

3.2. In 71% of LoViReT individuals we did not found replication-competent virus in peripheral blood and found limited provirus in anatomical compartments.

3.3. Low reservoirs are traditionally associated with early start of treatment, but we demonstrated that similar low reservoirs are found in early and chronic treated individuals.

3.4. LoViReT individuals presented a skewed distribution of the provirus among memory CD4$^+$ T cell subpopulations, with a high contribution of the short-live T$_{TM}$ and T$_{EM}$ cells in the total HIV-1 reservoir.

3.5. Despite an impaired reservoir establishment causing their low reservoirs, the absence of strong HIV-1 specific immune responses seems to jeopardize the control of HIV-1 replication in LoViReT individuals.

Objective 4: To evaluate the factors associated with the reduction of the viral reservoirs after an allogeneic hematopoietic stem cell transplant, specially using *CCR5* wild-type donors.

4.1. Allogeneic hematopoietic stem cell transplantation (HSCT) resulted by itself in a profound long-term reduction in the HIV-1 reservoir in the first 6 months after the transplant, including the cases using *CCR5* wild-type donors.

4.2. Three main transplant-associated factors may have contributed to the HIV-1 reduction, including the type of the stem cell donor, the time to full donor chimera, and the graft vs host disease. Altogether, it generates a "graft-versus-HIV effect" that seemed to be key to reduce the viral reservoir.

4.3. Declining HIV-1 specific antibodies were observed after the transplant in different grades, observing a case of seroreversion.

4.4. A non-controlled treatment interruption in an HIV-1-infected and transplanted individual result in an abrupt HIV-1 viral load rebound, with replenishment of the latent reservoir and a new seroconversion.

4.5. Allogeneic stem cell transplant caused a punctual and generalized potent immune response that extremely reduced the HIV-1 reservoirs by cell exchange done by the transplant itself. The absence of specific and sustained HIV-1 immune responses limited the possibility to control the HIV-1 rebound after treatment interruption.
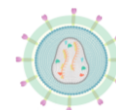
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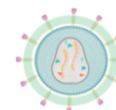24   Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM *et al.*

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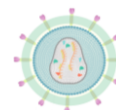
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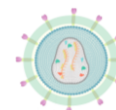
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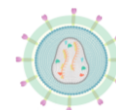
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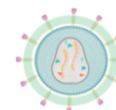
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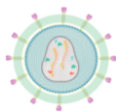
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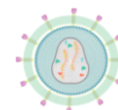
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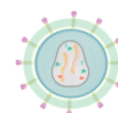
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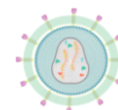
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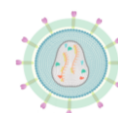
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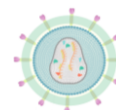
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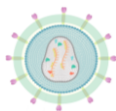
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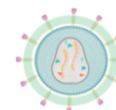
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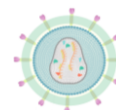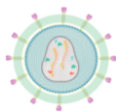
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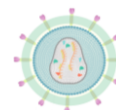
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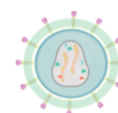
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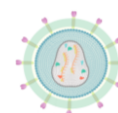
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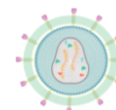342 Eberhard JM, Angin M, Passaes C, Salgado M, Monceaux V, Knops E *et al.*

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CHAPTER 7. PUBLICATIONS

1.   Benet S; <u>Gálvez C*</u>; Drobniewski F*; Kontsevaya I*; Arias L*; Monguió-Tortajada M*; Erkizia I; Urrea V; Ong R-Y; Luquin M; Dupont M; Chojnacki J; Dalmau J; Cardona P; Neyrolles O; Lugo-Villarino G; Vérollet C; Julián E; Furrer H; Günthard HF; Crocker PR; Tapia G; Borràs FE; Fellay J; McLaren PJ; Telenti A; Cardona P-J; Clotet B; Vilaplana C; Martinez-Picado J and Izquierdo-Useros N. **Dissemination of Mycobacterium tuberculosis is associated to a SIGLEC1 null variant that limits antigen exchange via trafficking extracellular vesicles.** [Submitted]

2.   Duran-Castells C, Llano A, Kawana-Tachikawa A, Prats A, Kobayashi-Ishihara M, Oriol-Tordera B, Penya R, <u>Gálvez C</u>, Clotet B, Riveira E, Ballana E, Prado J, Martinez-Picado J, Sanchez J, Mothe B, Hartigan-O'Connor D, Wyss-Coray T, Meyerhans A, Gisslen M, Price RW, Soriano-Mas C, Muñoz-Moreno JA, Brander C*, Ruiz-Riol M*. **SIRTUIN2 impacts HIV infection and neurological dysfunction.** [Submitted]

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CHAPTER 8. ACKNOWLEDGEMENTS

Y ha llegado la última parte de la tesis, y no por ello menos importante, la de agradecimientos ya que habría sido imposible hacerlo sin todos vosotros. Y aunque yo no soy mucho de expresar mis sentimientos ¡ahí va!

Me gustaría empezar por **Javier**, por darme la oportunidad de poder hacer el doctorado en su grupo y por haber sido el mejor jefe de grupo y director que podría haber esperado. No solo ha estado ahí científicamente, sino que también se ha preocupado a nivel personal en los momentos en los que he necesitado. Y por supuesto después me gustaría agradecerle a **Maria**, mi codirectora, que ha estado día a día conmigo ayudándome en todo lo posible (incluso dejándome su sofá sin apenas conocerme jeje). Y por todos esos momentos compartidos en p3 manejando miles y miles de millones de células (hasta crear "seres" macroscópicos), incluso a altas horas, pero siempre con una sonrisa. Y además, por haber confiado siempre en mí, lo cual le agradezco muchísimo.

Y ahora toca el turno de l@s GREC@S, ese gran equipo de personas que ha estado conmigo durante todos estos años. Me gustaría empezar por **Judith**, nuestra super Project manager. Aunque empezó amenazándome de que si no bebía Jägger no podría hacer la tesis en el grupo, al final se convirtió en un gran apoyo y amiga, no solo en el trabajo sino también fuera de él. Hemos compartido mil momentos únicos como viajes, escapadas, rayos sexys, apariciones estelares, "ha dicho fucsia", bodas, enfados míos (sorry!) y un largo etcétera. Después viene el grupo de reservorios que poco a poco va creciendo. A **Mª Carmen**, porque siempre está cuando se la necesita y tiene respuestas (¡y orden!) para todo. A **Mari**, por estar siempre dispuesta a ayudarme a pesar de que le haga ir a toda pastilla y la estrese un poco. A **Sílvia**, la deportista del grupo, por transmitir tan buen royo y por dejarme usar parte de sus resultados en esta tesis jeje. A **Ángel**, la incorporación más nueva del *reservoir team*, que viene pisando fuerte y siempre está dispuesto a darte una supercontestación a cualquier pregunta. Y también a **Sara**, aunque ya no esté en Irsi, siempre estaba dispuesta a ayudarme y aún lo sigue estando cuando la necesitas. Y ahora, pasando al resto del grupo…empezando por **Itziar**, nuestra experta en citómetros que siempre que lo he necesitado ha estado ahí para ayudarme con muchísima paciencia, mil gracias. A **Xabi**, mi compi de atrás, por compartir momentos de laboratorio y también marujiles
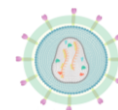
de Thermomix, charlas de perritos y marujeos de los que sino yo no me enteraría jeje. A **Patricia**, porque siempre que he necesitado su ayuda ha estado ahí sin dudarlo. To **Jakub**, for his enormous wisdom in microscopy and for sharing some THP-1 with me. Y por supuesto a nuestro gran bioestadístico, **Víctor**, aunque a veces le pidamos cosas imposibles siempre acabamos teniendo unos resultados maravillosos. Y aunque ya no formen parte de GREC y ahora sean los PISTA me gustaría incluirlos en esta sección. A **Núria**, por su gran sabiduría y apoyo durante toda esta estancia por Irsi, espero que con tu nuevo grupo tengas resultados increíbles, no me cabe duda. A **Dani**, el tarotista, bailarín y experto en Ébola del grupo, gracias por saber alegrar el día a cualquiera. Espero que te vaya genial en tu nueva etapa de doctor (allá donde sea) cuando todo esto acabe. Y a **Susana**, la médico del grupo siempre dispuesta a ayudar en cualquier dolencia tanto mía como de los míos, eres genial! Y por el currazo que te pegaste llamando y viendo a pacientes de LoViReT estando a punto de explotar, sin ti no hubiera sido posible. Y también por haber compartido tantos momentos fuera de Irsi con los *Gossip Pizza* y tu preciosa Martina a la que ya sabes que adoro (me falta conocer al pequeño Marc, que seguro que también me enamorará al instante).
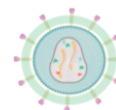
También me gustaría agradecer a algunas personas fueras de GREC. A **Óscar**, que compartió conmigo el primer artículo en solitario y aunque nos costó sudores y lágrimas al final quedó muy resultón. También a **Julià**, por ser el jefe con más marcha que he conocido, no me olvido de la noche en el Irish Rober jejeje. A **Júlia Garcia**, mi antigua compañera de mesa, por ser tan accesible y por la grata colaboración en el proyecto del pembro. A **Roger Badia**, que siempre está dispuesto a sacar una sonrisa o meterse contigo cuando te ve por P3. A **Bea**, por compartir todos sus conocimientos sobre en ensayos clínicos y VIH en general, y por ser una pieza fundamental para llevar a cabo el reclutamiento de los pacientes del estudio LoViReT, muchísimas gracias (y también por haber sido parte de mi comisión de seguimiento durante estos 4 años). A **Marta Massanella**, que ya desde la distancia en Montreal intentó ayudarme a poner a punto el TILDA, por esas comidas compartidas pre-pandemia, y por aceptar sin dudarlo a formar parte de mi tribunal de tesis. A **Samandhy**, que siempre tiene una sonrisa y buenas palabras, aún sigo sin saber dónde están los pellets que te debía en mi sueño jaja. A **Marisa**,

que cuando era mi compi de ciudad no dudó en hacerme de taxi. A **Eli** que a pesar de llamarme lunática y asombrarse siempre que me ve con chaqueta de borreguito en verano, sé que algo de aprecio me tiene. A **Marta Ruiz** y **Christian Brander**, por saber aprovechar las determinaciones de proviral como nadie para diferentes artículos. A **Lucia Gómez**, **Rafi** y **Cristina Ramírez**, por su gran labor en el equipo de muestras ya que sin ellas no podrían llevarse a cabo todos los estudios que se hacen, y además lo hacen con una sonrisa. Al equipo de **comunicación**, por ayudarnos a dar a conocer nuestros resultados al mundo a pesar de que yo odie tener que ponerme delante de una cámara. A **Penélope**, **Cristina Mesa** y **Arnau**, por su gran labor administrativa y solucionar cualquier problema que surja de pedidos, envíos, etc. A **Lidia**, por su gran labor como Lab Manager general de Irsi, sin ti las cosas no funcionarían igual. Y a **Julián**, nuestro informático, por solucionar con rapidez siempre que tenemos algún problema. También me gustaría agradecer a **Lourdes** y **Ventura**, ya que sin su gestión y saber hacer no habría sido posible los 25 años que lleva en pie IrsiCaixa. Y en general, aunque no estéis mencionados, a todas las personas que forman parte de esta gran familia que es IrsiCaixa.

También quisiera agradecer a todo el equipo de Lluita ya que siempre me han ayudado cuando les he necesitado, **Jordi**, **Albert**, **Andrea**, **Lucía**, **Boris**, **Josep Maria Llibre**, **Aroa**… Y a los servicios del campus de Can Ruti. A **Vicky**, de micro, por ser tan amable y estar siempre dispuesta a hacer nuestras determinaciones de anticuerpos. Y a **Marco**, en Citometria, por ser tan crack en lo suyo y haberme ayudado tantísimo en todos los estudios en los que la citometría estaba involucrada, sobre todo aquel verano en el que nos peleamos con el Poltergeist de los anticuerpos y que a día de hoy sigue siendo un gran misterio sin resolver.

I would like to thank the people from Imperial College in London where I spent for almost two months doing Siglec-1 PCRs like crazy. Specially to **Francis**, the PI, **Gurpreet** and **Irina** for making my stay so pleasant and easy. Also, to all the people from Ragon Institute in Boston where I did my PhD internship (and surprisingly I did not die despite the thermometer reached -18ºC). **Galit** for giving me the opportunity to stay at your lab and learn all those fancy assays you have, and for your enthusiasm every time I talked to you. To **Madeline** for all her incredible help with the tedious process of the visa and the initial paperwork at Ragon. To **Matt**, who

patiently taught me all the techniques despite having himself a lot of work. Y por supuesto a **Marianna**, que fue un gran apoyo durante mi estancia en Boston y compartimos muy buenos momentos.

Para acabar quisiera agradecer a mis padres, **Manolo** y **Montse,** sin los cuales a día de hoy no podría haber realizado esta tesis. A pesar de que mi madre no se entere muy bien de lo que he estudiado y de lo que hago, siempre me pregunta qué estoy haciendo y que le explique de que van mis experimentos. A mi hermana **Jen**, por ser tan buena hermana conmigo, por hablar con tanto orgullo de mi con la gente y por estar ahí siempre que la he necesitado. A mi tía **Nieves**, que ha estado ahí desde que nací ayudándome en todo. Gracias también a mi familia política, mis suegros **Paco** y **Mari**, y mis cuñados **Lorena** y **Samir**, por estar siempre ahí y confiar en que tendré respuestas a todas sus dudas científicas, especialmente en tiempos de COVID. Y también a los pequeñajos **Edgar** y **Elan**, aunque aún no sepan a lo que me dedico yo intento explicárselo para que vayan pillando algo y alguno de ellos quiera seguir mis pasos.

Gracias también a **Cristian**, que ha sido un gran apoyo y me ha aguantado durante todos estos años de tesis, ya que le he martirizado ensayando mis presentaciones y pasándole todos mis artículos para que se los lea (aunque se queje de que es imposible entender nada con tantas siglas).

También, aunque no puedan leerlo, a mis compañeros peludos **Darko** y **Enzo** que me han acompañado durante parte de la escritura de esta tesis sin despegarse de mi en un solo momento.

Por último, gracias a todos los **pacientes** que han accedido a colaborar desinteresadamente en todos y cada uno de los estudios que conforman esta tesis, sin ellos hubiera sido imposible realizar esta tesis.

¡MUCHAS GRACIAS!