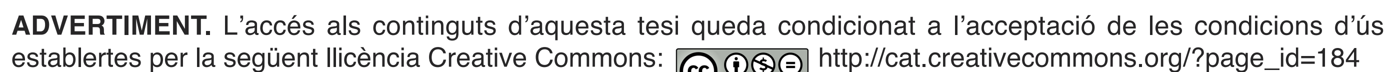
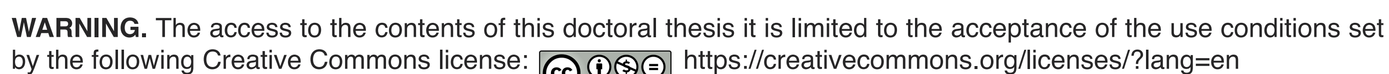
ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons:  http://cat.creativecommons.org/?page_id=184

ADVERTENCIA. El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons: http://es.creativecommons.org/blog/licencias/

WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

Department of Biochemistry and Molecular Biology
Faculty of Medicine
Universitat Autònoma de Barcelona

Evaluation of clinical strategies to cure HIV-1 infection in patients receiving antiretroviral therapy

Sara Morón-López

IrsiCaixa – AIDS Research Institute
Hospital Universitari Germans Trias i Pujol
2016

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

Director: Dr. Javier Martinez-Picado
Tutor: Dr. Xavier Daura Ribera

Cover & CD design: Jordi Leiva Maturana, 2016

Cover & CD illustrations: Jordi Leiva Maturana & Sara Morón-López, 2016

Bookmark design: LLARdigital, 2016

Sara Morón-López was supported by grant 2013FI_B 00275 (Agència de Gestió d'Ajuts Universitaris i de Recerca) from Generalitat de Catalunya. The content of this thesis was partly funded by Gilead, the Spanish Ministry of Health and Social Policy under the project 'The TRI-ANTiretroviral and coadjunctive ThErapies for HIV-associated neurocognitive impairment study (TRIANT-TE)' [EC10-320] to 'Lluita Contra la Sida' Foundation, NHS Trust, and amfAR grant 108930-56-RGRL. Additional support was provided by the Spanish Science and Innovation Ministry (grant SAF2013-49042-R) and the Spanish AIDS network "Red Temática Cooperativa de Investigación en SIDA" (RD12-0017-0002).

The printing of this thesis was made possible with the financial aid of the Universitat Autònoma de Barcelona.

IrsiCaixa
Institut de Recerca de la Sida

El **Dr. Javier Martinez-Picado**, investigador principal i professor de recerca ICREA a l'Institut de Recerca de la SIDA – IrsiCaixa,

Certifica:

Que el treball experimental i la redacció de la memòria de la Tesi doctoral titulada "Evaluation of clinical strategies to cure HIV-1 infection in patients receiving antiretroviral therapy" han estat realitzades per la Sara Morón-López sota la seva direcció i considera que és apta per a ser presentada per optar al grau de Doctora en Bioquímica, Biologia Molecular i Biomedicina per la Universitat Autònoma de Barcelona.

I per tal que quedi constància, signa aquest document.

Badalona, el 14 de juliol de 2016.

Dr. Javier Martinez-Picado

El **Dr. Xavier Daura Ribera**, director de l'Institut de Biotecnologia i de Biomedicina "Vicent Villar Palasí" i investigador ICREA a l'Institut de Biotecnologia i de Biomedicina "Vicent Villar Palasí"

Certifica:

Que el treball experimental i la redacció de la memòria de la Tesi doctoral titulada "Evaluation of clinical strategies to cure HIV-1 infection in patients receiving antiretroviral therapy" han estat realitzades per la Sara Morón-López sota la seva tutoria i considera que és apta per a ser presentada per optar al grau de Doctora en Bioquímica, Biologia Molecular i Biomedicina per la Universitat Autònoma de Barcelona.

I per tal que quedi constància, signa aquest document.

Badalona, el 14 de juliol de 2016.

Dr. Xavier Daura Ribera

A mis padres,
por su apoyo incondicional

A mi abuelo

“Si no conozco una cosa,
la investigaré”

Louis Pasteur

ABBREVIATIONS

1-LTR	1-Long Terminal Repeat
2-LTR	2-Long Terminal Repeat
3TC	Lamivudine
ABC	ABaCavir
AF700	Alexa FluorTM 700
AIDS	Acquired Immunodeficiency Syndrome
APC	AlloPhycoCyanin
APC-Cy7	AlloPhycoCyanin-Cy7 conjugate
APOBEC3G	APOlipoprotein B messenger ribonucleic acid editing Enzyme Catalytic polypeptide-like 3G
ART	AntiRetroviral Therapy
AZT	AZidoThymidine
bp	base pair
BST	Banc de Sang i Teixits
BST2	Bone marrow STromal cell antigen 2
BV421	Brilliant Violet 421
BV605	Brilliant Violet 605
CA	CApsid
CA-RNA	Cell-Associated RiboNucleic Acid
CB	Cord Blood
CBU	Cord Blood Unit
CCR5	C-C chemokine Receptor type 5
CCR7	C-C chemokine Receptor type 7
CD123	Interleukin-3 receptor alpha chain
CD14	Cluster of Differentiation 14
CD15	3-fucosyl-N-acetyl-lactosamine
CD16	Cluster of Differentiation 16
CD19	Cluster of Differentiation 19
CD235a	Glycophorin A

CD27	Traf-linked tumor necrosis factor receptor 27
CD3	Cluster of Differentiation 3
CD34	Cluster of Differentiation 34
CD36	Cluster of Differentiation 36
CD38	Cluster of Differentiation 38
CD4	Cluster of Differentiation 4
CD45	Cluster of Differentiation 45
CD45RA	Cluster of Differentiation 45RA
CD56	Neural cell adhesion molecule
CD61	Integrin beta-3
CD8	Cluster of Differentiation 8
CDC	Center for Disease Control
cDNA	complementary DeoxyriboNucleic Acid
CEIC	Comité Ético de Investigación Clínica
CFDA	CarboxyFluorescein DiAcetate succinimidyl ester
CFU	Colony Forming Units
CHOP-R	Cyclophosphamide Doxorubicin Vincristine Prednisone Rituximab non-Hodgkin's lymphoma treatment
CMV	CytoMegaloVirus
CRF	Circulating Recombinant Forms
CRISPR/Cas9	ClusteRed Interspaced Short Palindromic Repeats/CRISPR-associated protein-9 nuclease
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CTLs	Cytotoxic T Lymphocytes
CXCR4	C-X-C chemokine Receptor type 4
ddPCR	droplet digital Polymerase Chain Reaction
DLBCL	Diffuse Large B-Cell Lymphoma
DNA	DeoxyriboNucleic Acid
DNase	DeoxyriboNuclease
DNMTs	DeoxyriboNucleic acid MethylTransferases
dNTPs	deoxyNucleoTides
dsDNA	double-stranded DeoxyriboNucleic Acid

EBMT	European Society for Bone Marrow Transplantation
EBV	Epstein-Barr Virus
EDTA	EthyleneDiamineTetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked Immunosorbent Spot
env	Viral envelope glycoprotein
ESCRT	Endosomal Sorting Complexes Required to Transport
ESHAP-R	Etoposide Methylprednisolone Cytarabine Cisplatin Rituximab non-Hodgkin's lymphoma treatment
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein
FMO	Fluorescence Minus One
FTC	Emtricitabine
GALT	Gut-Associated Lymphoid Tissue
GEMOX	GEMcitabine OXaliplatin lymphoma treatment
GM	Granulocyte Macrophages
GSK-3	Glycogen Synthase Kinase 3
GTAC	Gene Therapy Advisory Committee
GUSB	Glucuronidase beta
HCV	Hepatitis C Virus
HDACs	Histone DeACetylases
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HLA	Human Leukocyte Antigen
HLA-DR	Human Leukocyte Antigen-DR
HMTs	Histone MethylTransferases
HPTN 052	HIV Prevention Trials Network 052 trial
HSCT	Hematopoietic Stem Cell Transplantation

IFN	InterFeroN
IFNα	InterFeroN alpha
IFNγ	InterFeroN gamma
IL-2	InterLeukin-2
IL-7	InterLeukin-7
IL-15	InterLeukin-15
IL-21	InterLeukin-21
InIs	Integrase Inhibitors
IQR	InterQuartile Range
ISG	Interferon-Stimulated Gene
IUPM	Infectious Units Per Million cells
J-lat cells	Jurkat latently infected cell line
LAV	Lympho-Adenopathy-associated Virus
LEF-1	Lymphoid Enhancer-binding Factor 1
LOD	Limit Of Detection
LRA	Latency-Reversing Agent
LTR	Long Terminal Repeat
MA	MAtrix
MHC	Major Histocompatibility Complex
msRNA	multi-spliced RiboNucleic Acid
MT-4 cells	MT-4 human T cell leukemia derived cell line
MVA	Modified Vaccinia virus Ankara
MVA.HIVconsv	HIVconsv immunogen vectored by modified vaccinia virus Ankara
mVOA	murine Viral Outgrowth Assay
NC	NucleoCapsid
ND	Non-Determined
NF-κB	Nuclear Factor kappa B
NG	No Growth
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors

NRTIs	Nucleoside-analog Reverse Transcriptase Inhibitors
Nuc	Nucleosome
NVP	NeViraPine
OKT3	Anti-cluster of differentiation 3 antibody
P-TEFb	Positive Transcription Elongation Factor b
P/S	Penicillin/Streptomycin
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed cell Death protein 1
PD-L1	Programmed cell Death protein Ligand 1
PE-CF594	PhycoErythrin alexa fluor 594 conjugate
PE-Cy7	PhycoErythrin-Cy7 conjugate
PerCP	Peridinin Chlorophyll Protein
pfu	plaque-forming units
PHA	PhytoHemAgglutinin
PIC	Pre-Integration Complex
PIs	Protease Inhibitors
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
qPCR	quantitative Polymerase Chain Reaction
qVOA	quantitative Viral Outgrowth Assay
RAL	Raltegravir
RBV	RiBaVirin
RhCMV	Rhesus CytoMegaloVirus
RNA	RiboNucleic Acid
Rnase	Ribonuclease
RPMI	Rowell Park Memorial Institute
RPP30	Ribonuclease P/MRP subunit p30
RT	Reverse Transcriptase
RT-ddPCR	Reverse Transcription droplet digital Polymerase Chain Re-

	action
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
SCA	Single-Copy Assay
SFU	Spot Forming Units
Siglec-1	Sialic acid binding immunoglobulin-like lectin 1
SIV	Simian Immunodeficiency Virus
ssRNA	single-stranded RiboNucleic Acid
START	Strategic Timing of AntiRetroviral Treatment study
TALENs	Transcription Activator-Like Effector Nucleases
TBP	TATA-Binding Protein
TCF	T-cell factor
TCF-1	T-cell factor-1
TCF-4	T-cell factor-4
TCGF	T-cell growth factors
T_{CM}	$T_{\text{Central Memory}}$
TCR	T Cell Receptor
TCRγ/δ	T Cell Receptor gamma/delta
TDF	Tenofovir
T_{EM}	$T_{\text{Effector Memory}}$
T_{EMRA}	$T_{\text{terminally differentiated Effector Memory RA+}}$
T_{FH}	$T_{\text{Follicular Helper}}$
TRIANT-TE	TRI-ANTiretroviral and coadjunctive ThErapies for HIV-associated neurocognitive impairment study
TILDA	Tat/rev-Induced Limiting Dilution Assay
TMA	Transcription-Mediated Amplification
T_{MM}	$T_{\text{Migratory Memory}}$
T_N	$T_{\text{Naïve}}$
TNC	Total Nucleated Cells
TPHD	Third-Party Haploidentical Donor
TRIM22	TRIpartite Motif-containing 22
TRIM5α	TRIpartite Motif-containing 5 alpha

T_{RM}	T tissue Resident Memory
tRNA	transfer RiboNucleic Acid
T_{SCM}	T Stem Cell Memory
TSS	Transcriptional Start Site
T_{TD}	T Terminally Differentiated
T_{TM}	T Transitional Memory
U87 cells	Human primary glioblastoma cell line
U87-CD4-CCR5	Human glioblastoma cell line CD4+CCR5+
U87-CD4-CXCR4	Human glioblastoma cell line CD4+CXCR4+
usRNA	unspliced RiboNucleic Acid
VISCONTI	Virological and Immunological Studies in CONtrollers after Treatment Interruption
VL	Viral Load
WT	Wild Type
ZFN	Zinc-Finger Nuclease

TABLE OF CONTENTS

SUMMARY, RESUMEN & RESUM 25

THESIS PRESENTATION 33

CHAPTER 1. INTRODUCTION 37

1.1. Human Immunodeficiency Virus 39

1.1.1. Origin, classification and current epidemic situation 39

1.1.2. HIV-1 structure and genome 40

1.1.3. HIV-1 replication cycle 42

1.2. Natural course of HIV-1 infection 44

1.3. Antiretroviral therapy and persistence of HIV-1 infection 46

1.4. Assays to quantify the HIV-1 reservoir 49

1.4.1. Total or integrated reservoir and residual replication – qPCR or ddPCR and Alu-PCR 50

1.4.2. HIV-1 expression – RT-qPCR or RT-ddPCR 51

1.4.3. Residual plasma viremia – ultrasensitive viral load 52

1.4.4. Replication-competent reservoir – qVOA and mVOA 53

1.4.5. Inducible reservoir – TILDA 54

1.5. Therapeutic approaches for HIV-1 cure 55

1.5.1. Treatment optimization (intensification, switching and early-treatment) 55

1.5.2. Immunotherapy 57

1.5.3. Latency reactivation 58

1.5.4. Therapeutic vaccination 61

1.5.5. Cellular or gene therapy 63

CHAPTER 2. HYPOTHESIS AND AIMS 67

CHAPTER 3. CLINICAL TRIAL RESULTS 71

CHAPTER 3A. Immunotherapy 73

Short-term treatment with interferon alpha diminishes expression of HIV-1 and reduces $CD4^+$ T-cell activation in patients coinfected with HIV and hepatitis C virus and receiving antiretroviral therapy 74

3A.0. Presentation 75

3A.1. Introduction 76

3A.2. Material and Methods 78

3A.2.1 Patients 78

3A.2.2. Peripheral Blood Mononuclear Cells (PBMCs) isolation and preservation 78

3A.2.3. CD4$^+$ T-cell purification 78
3A.2.4. Cell-associated HIV-1 mRNA determination 79
3A.2.5. Ultrasensitive plasma viremia quantification 81
3A.2.6. Quantitative viral outgrowth assay (qVOA) determination 82
3A.2.7. Proviral HIV-1 DNA quantification 84
3A.2.8. HIV-1 2-LTR circles quantification 85
3A.2.9. mRNA expression of APOBEC3G, TRIM5α, BST2, and TRIM22 85
3A.2.10. Distribution and activation of T-cell subsets 86
3A.2.11. Statistical analysis 86
3A.3. Results 88
3A.3.1. Patients 88
3A.3.2. Cell-associated HIV-1 mRNA and residual viremia 88
3A.3.3. Replication-competent HIV-1 reservoir, total HIV-1 DNA, and 2-LTR DNA circles 90
3A.3.4. Expression of IFNα-stimulated genes (ISGs) 90
3A.3.5. Analysis of CD4$^+$ T-cell subsets, activation and exhaustion markers 93
3A.4. Discussion 95

CHAPTER 3B. Latency reactivation 97

Effect of lithium on HIV-1 expression and proviral reservoir size in the CD4$^+$ T cells of antiretroviral therapy suppressed patients 98

3B.0. Presentation 99
3B.1. Introduction 100
3B.2. Material and Methods 101
3B.2.1. Patients 101
3B.2.2. Cell-associated HIV-1 mRNA 101
3B.2.3. Proviral HIV-1 DNA 101
3B.2.4. Ultrasensitive plasma viremia 101
3B.2.5. Quantitative viral outgrowth assay (qVOA) 101
3B.2.6. Statistical analysis 102
3B.3. Results 103
3B.3.1. Patients 103
3B.3.2. Cell-associated HIV-1 mRNA and residual viremia 103
3B.3.3. Total HIV-1 DNA and replication-competent HIV-1 reservoir 103
3B.4. Discussion 106

CHAPTER 3C. Therapeutic vaccination 109

Evaluation of the immunogenicity and impact on the latent HIV-1 reservoir of a conserved region vaccine, MVA.HIVconsv, in antiretroviral therapy-treated subjects 110

3C.0. Presentation ... 111
3C.1. Introduction ... 112
3C.2. Material and Methods ... 114
3C.2.1. Patients ... 114
3C.2.2. Randomization and masking ... 114
3C.2.3. Vaccine and vaccination schedule ... 114
3C.2.4. Peptides ... 115
3C.2.5. Ex vivo IFN-γ Elispot assay ... 115
3C.2.6. Viral inhibition assay ... 116
3C.2.7. Quantification of HIV-1 proviral DNA in $CD4^+$ T cells ... 116
3C.2.8. Residual plasma viremia ... 116
3C.2.9. Statistical analysis ... 116
3C.3. Results ... 117
3C.3.1. Study participants and vaccinations ... 117
3C.3.2. Vaccine safety ... 118
3C.3.3. T cell responses to MVA.HIVconsv ... 119
3C.3.4. Effect of MVA.HIVconsv vaccinations on the HIV-1 reservoir ... 124
3C.4. Discussion ... 127

CHAPTER 3D. Cellular therapy ... 131

CCR5 Δ32 homozygous cord blood allogeneic transplantation in a patient with HIV: a case report ... 132

3B.0. Presentation ... 133
3D.1. Introduction ... 134
3D.2. Material and Methods ... 135
3D.2.1. Chimerism analysis ... 135
3D.2.2. CCR5 genotyping and viral tropism assay ... 135
3D.2.3. Virus Isolation and Sequence Analysis ... 135
3D.2.4. Reservoir parameters ... 135
3D.2.5. Semiquantitative PCR based detection ... 135
3D.2.6. Immune activation and Proliferation ... 136
3D.2.7. Cell Infectivity Assay ... 136
3D.3. Results ... 137
3D.3.1 Case report ... 137
3D.3.2. Viral Characteristics and Tropism ... 139
3D.3.3. Latent reservoir ... 140
3D.3.4. Cell recovery and Infectivity ... 141
3D.4. Discussion ... 143

CHAPTER 4. GLOBAL DISCUSSION AND PERSPECTIVES ... 147

CHAPTER 5. CONCLUSIONS 153

CHAPTER 6. REFERENCES 157

CHAPTER 7. PUBLICATIONS..187

CHAPTER 8. ACKNOWLEDGEMENTS 191

SUMMARY, RESUMEN & RESUM

The development of drugs that inhibit the human immunodeficiency virus type 1 (HIV-1) replication cycle, known as combination antiretroviral therapy (ART), was one of the major clinical successes of the 20th century. However, despite the fact that this treatment efficiently suppresses viral replication, improves the immune function and decreases the morbidity and mortality associated with the acquired immunodeficiency syndrome (AIDS), ART cannot cure this infection and has several limitations, as it: (i) cannot completely decrease the chronic inflammation and immunosenescence, (ii) cannot eradicate the latently infected cells, and (iii) requires adherence to daily drug regimens to avoid viral rebound, drug resistance and disease progression. Therefore, the development of new approaches to cure HIV-1 infection is a major necessity worldwide.

The strategies that are currently in evaluation are: treatment optimization, immunotherapy, latency reactivation, therapeutic vaccination, and cellular and gene therapy. In this context, the aim of this thesis is to evaluate the curative potential of four clinical trials based on: (i) immunotherapy with interferon-α (IFNα), (ii) latency reactivation with lithium carbonate, (iii) therapeutic vaccination with HIV-1 conserved region immunogen (MVA.HIVconsv), and (iv) cellular therapy with an hematopoietic stem cell transplantation (HSCT) using an HIV-resistant cord blood unit. All these studies were performed in chronically ART-suppressed individuals. For that purpose, we analyzed virological and immunological markers to quantify the HIV-1 reservoir and the immune function, using cellular and molecular biology methods.

Our results showed that immunotherapy with IFNα and administration of lithium could be used in combination with ART, since both act as viral repressors but cannot directly cure HIV-1 infection. Moreover, we observed that MVA.HIVconsv vaccine efficiently elicits HIV-specific cytotoxic immune responses against conserved regions of the HIV-1 genome. However, these responses are not sufficient to have an impact on the HIV-1 reservoir. Therefore, the vaccine should be administered in combination with a latency-reversing agent (LRA). Finally, there were signs of recovery from the HIV-1 infection in the individual treated with the HSCT using an HIV-resistant cord blood unit. Unfortunately, it was impossible to confirm this ob-

servation in samples 3 months after the transplant, time at which the patient passed away due to the quick progression of a lymphoma.

In the future, more clinical trials based on these or other novel strategies should be performed to find the optimal strategies that target the heterogeneous population of infected individuals, which ultimately will allow for the eradication of this pandemic.

El desarrollo de fármacos que inhiben el ciclo de replicación del virus de la inmunodeficiencia humana tipo 1 (VIH-1), conocidos como terapia antirretroviral combinada (TAR), fue uno de los mayores éxitos clínicos del siglo XX. Sin embargo, a pesar de que este tratamiento suprime eficientemente la replicación viral, mejora la función inmunológica y disminuye la morbilidad y mortalidad asociadas al síndrome de la inmunodeficiencia adquirida (SIDA), la TAR no es capaz de curar esta infección y tiene diversas limitaciones: (i) no puede reducir completamente la inflamación crónica ni la inmunosenescencia, (ii) no puede erradicar las células latentemente infectadas, y (iii) requiere adherencia diaria al tratamiento para evitar el rebote viral, la resistencia a fármacos y la progresión de la enfermedad. Por tanto, el desarrollo de nuevas estrategias con el objetivo de curar la infección por VIH-1 es un requisito ineludible a nivel mundial.

Las estrategias que están siendo actualmente evaluadas son: la optimización del tratamiento antirretroviral, la inmunoterapia, la reactivación de latencia, la vacunación terapéutica, y la terapia celular y génica. En este contexto, el objetivo de esta tesis es evaluar el potencial curativo de cuatro estudios clínicos basados en: (i) inmunoterapia con interferón-α (IFNα), (ii) reactivación de latencia con carbonato de litio, (iii) vacunación terapéutica con inmunógeno de regiones conservadas de VIH-1 (MVA.HIVconsv), y (iv) terapia celular con trasplante de células madre hematopoyéticas usando células de cordón umbilical resistentes a la infección por VIH-1. Todos estos estudios se realizaron en individuos tratados en fase crónica y con viremia suprimida. Para ello se analizaron marcadores virológicos e inmunológicos para cuantificar el reservorio de VIH-1 y la función inmunológica, utilizando métodos de biología celular y molecular.

Los resultados mostraron que tanto la inmunoterapia con IFNα como la administración de litio se podrían usar como implementación del tratamiento, en combinación con la TAR, ya que ambos actúan como represores virales. Además, se observó que la vacuna de región conservada MVA.HIVconsv genera respuestas citotóxicas específicas contra regiones conservadas del genoma de VIH-1; sin embargo, estas respuestas no son suficientes para afectar al reservorio de VIH-1, de modo que esta vacunación debería ser administrada en combinación con agentes reactivadores de la latencia. Finalmente, el trasplante alogénico con célu-

las de cordón umbilical resistente a la infección por VIH-1 mostró que el paciente infectado con VIH-1 tratado podría haberse curado mediante esta estrategia terapéutica. Desafortunadamente, el paciente falleció tres meses después del trasplante debido a una rápida progresión del linfoma que padecía, y no fue posible confirmar esta hipótesis con análisis posteriores.

Por consiguiente, es necesario realizar más estudios clínicos basados en éstas o nuevas estrategias terapéuticas para conseguir un tratamiento, de aplicación generalizada para todos los individuos infectados por VIH, el cual permita conseguir curar definitivamente esta pandemia.

El desenvolupament de fàrmacs que inhibeixen el cicle de replicació del virus de la immunodeficiència humana tipus 1 (VIH-1), coneguts com teràpia antiretroviral combinada (TAR), va ser un dels majors èxits clínics del segle XX. Tanmateix, malgrat que el tractament suprimeix eficientment la replicació viral, millora la funció immunitària i disminueix la morbiditat y mortalitat associades a la síndrome de la immunodeficiència humana (SIDA), la TAR no és capaç de curar aquesta infecció i presenta diverses limitacions: (i) no pot reduir completament la inflamació crònica ni la immunosenescència, (ii) no pot eradicar les cèl·lules latentment infectades, i (iii) requereix adherència diària al tractament per evitar el rebot viral, la resistència a fàrmacs i la progressió de la malaltia. Per tant, el desenvolupament de noves estratègies amb l'objectiu de curar la infecció per VIH-1 és un requisit ineludible a nivell mundial.

Les estratègies que estan sent avaluades actualment són: la optimització del tractament, la immunoteràpia, la reactivació de la latència, la vacunació terapèutica i la teràpia cel·lular i gènica. En aquest context, l'objectiu d'aquesta tesi és avaluar el potencial curatiu de quatre estudis clínics basats en: (i) immunoteràpia amb interferó-α (IFNα), (ii) reactivació de latència amb carbonat de liti, (iii) vacunació terapèutica amb immunogen de regions conservades de VIH-1 (MVA.HIVconsv), i (iv) teràpia cel·lular amb trasplantament de cèl·lules mare hematopoètiques utilitzant cèl·lules de cordó umbilical resistent a la infecció per VIH-1. Tots aquests estudis es van realitzar en individus tractats en fase crònica i amb virèmia suprimida. En els estudis avaluats es van analitzar marcadors virològics i immunològics per quantificar el reservori de VIH-1 i la funció immunològica, utilitzant mètodes de biologia cel·lular i molecular.

Els resultats van mostrar que tant la immunoteràpia amb IFNα com l'administració de liti es podrien utilitzar com a implementació del tractament, en combinació amb la TAR, ja que ambdós actuen com a repressors virals. A més, es va observar que la vacuna de regió conservada MVA.HIVconsv genera respostes citotòxiques específiques contra regions conservades del genoma de VIH-1; ara bé, aquestes respostes no són suficients com per afectar al reservori del VIH-1, de manera que aquesta vacunació hauria de ser administrada en combinació amb agents reactivadors de la latència. Finalment, el trasplantament de cèl·lules

de cordó umbilical resistent a la infecció per VIH-1 va mostrar que el pacient infectat per VIH-1 tractat podria haver-se curat mitjançant aquesta estratègia terapèutica. Malauradament, el pacient va morir tres mesos després del trasplantament degut a una ràpida progressió del limfoma que patia, i no va ser possible confirmar aquesta hipòtesi amb anàlisis posteriors.

Per tant, és necessari realitzar més estudis clínics basats en aquestes o noves estratègies terapèutiques per aconseguir un tractament, d'aplicació generalitzada per a tots els individus infectats per VIH, el qual permeti aconseguir curar definitivament aquesta pandèmia.

THESIS PRESENTATION

The proposed thesis, including a complete introduction, has been written with the necessary knowledge to understand the data shown. Four chapters of results –corresponding to each of the evaluated studies–, a global discussion and future perspectives, and the conclusions obtained from this work are presented. The "Results" chapter includes a specific introduction, material and methods, results and discussion for each study included in this thesis. The reason for not following the standard format is because we have considered that this work would be easier to understand and follow, and its presentation would be more appropriate with this alternative outline.

CHAPTER 1. INTRODUCTION

1.1. Human Immunodeficiency Virus

1.1.1. Origin, classification and current epidemic situation

In 1981, clinicians from Los Angeles and the Centers for Disease Control (CDC) in the United States of America reported the first cases of *Pneumocystis jiroveci* pneumonia and candidiasis among previously healthy young men (1,2). The patients presented a depletion of $CD4^+$ T cells, which compromised their immune system, and unusual infections or/and cancers, such as Kaposi's sarcoma. Later, this clinical symptomatology became known as the Acquired Immunodeficiency Syndrome (AIDS), and in 1983, the pathological agent known as lymphoadenopathy-associated virus (LAV) was isolated from a lymph node biopsy of an AIDS patient and identified as the etiological agent causing this disease (3). In 1984, two research groups confirmed this novel retrovirus as the causative agent of AIDS (4,5). Later, this virus was denominated as Human Immunodeficiency Virus (HIV) by the International Committee on the Taxonomy of Viruses (6).

Current studies estimate that HIV first infected humans in the 1920s in Kinshasa (7–9) through multiple zoonotic infections, or cross-species transmissions, from simian immunodeficiency virus (SIV), which infects non-human primates (10). Moreover, other studies revealed two distinct lentiviruses with different SIV ancestors as the cause of AIDS in humans: human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) (11). HIV-1 is phylogenetically related to SIVcpz, which was isolated from the subspecies *Pan troglodytes troglodytes* (12), whereas HIV-2 is more closely associated with SIVsm, which was identified in sooty mangabeys *Cercocebus atys* (13).

HIV, including the HIV-1 and HIV-2 variants, is a member of the group VI of reverse transcribing viruses, *Retroviridae* family, *Orthoretroviridae* subfamily, and *Lentivirus* genus (14). The HIV species are differentiated by their replicative and pathogenic capacity, virus evolution and target of infection. HIV-1 is the most prevalent type of HIV, which is spread over the world, whereas HIV-2 mainly remains geographically restricted to West Africa. Their different distribution could be explained by the poor capacity for transmission and less pathogenic course of infection of HIV-2 (15). HIV-1 comprises four different groups, M (major or main), N

(non-M, non-O), O (outlier), and P (putative). HIV-2 comprises the groups A, B, C, D, E, F, G and the recombinant AB. HIV-1 M and O groups, as well as the HIV-2 A and B, have been the only ones to expand at a pandemic level and infect millions of people. Moreover, the M group accounts for the majority of worldwide infections and can be subdivided into different clades: A, B, C, D, F, G, H, J, K and Circulating Recombinant Forms (CRF) (16,17).

In 2014, it was estimated that about 37 million people were living with HIV, 2 million were newly infected and more than 1 million died from AIDS-related illnesses. Among the people living with HIV, almost 13.6 and 15.8 million people were receiving antiretroviral therapy (ART), in June 2014 and 2015 respectively (18). Therefore, as the prevalence of HIV infection raises yearly, mortality from AIDS-related illnesses decreases, due to the continuous increase in the access to ART. Nevertheless, the infection causing this pandemic remains incurable.

1.1.2. HIV-1 structure and genome

The HIV-1 is a single-stranded RNA (ssRNA) virus whose genome is composed of two positive ssRNA chains of approximately 9.8 kb in length (19). The viral genome is flanked by long terminal repeats (LTR) and encodes for nine genes classified according to its protein function (Fig.1).

- Major structural and functional genes:
    - *GAG* codifies for the Gag polyprotein, which is processed during virion maturation, generating the structural proteins p17 or matrix (MA), p24 or capsid (CA), p7 or nucleocapsid (NC) and p6.
    - *POL* codes for the viral enzymes reverse transcriptase (RT), integrase and protease.
    - *ENV* encodes for the polyprotein gp160, precursor of gp120 and gp41. These proteins are part of the viral envelope and are responsible for the target cell recognition, attachment and fusion.

- Regulatory genes:
    - *TAT* and *REV* code for the regulatory proteins Tat and Rev that control transcriptional and posttranscriptional HIV-1 gene expression.

- Accessory genes:
    - *VIF, VPR, VPU* and *NEF* encode for the accessory proteins Vif, Vpr, Vpu and Nef, whose functions are not essential for the HIV-1 replication but increase the efficiency of infection *in vivo*.

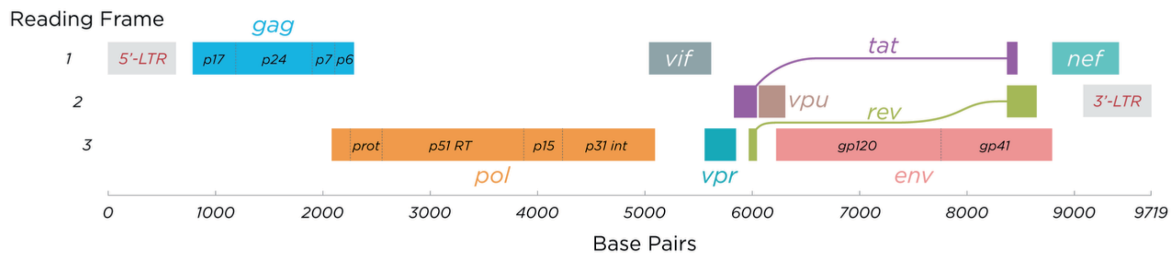


Figure 1. Schematic diagram of the HIV-1 genome showing gene names in italics and protein names in boxes organized according to their order in the open reading frame. Provided by Splettstoesser T. (20).

HIV-1 virions are spherical particles of 120-150 nm in diameter formed by a nucleocapsid (NC), a capsid (CA) and a matrix (MA), covered by a lipid bilayer from the host cell membrane (Fig.2).

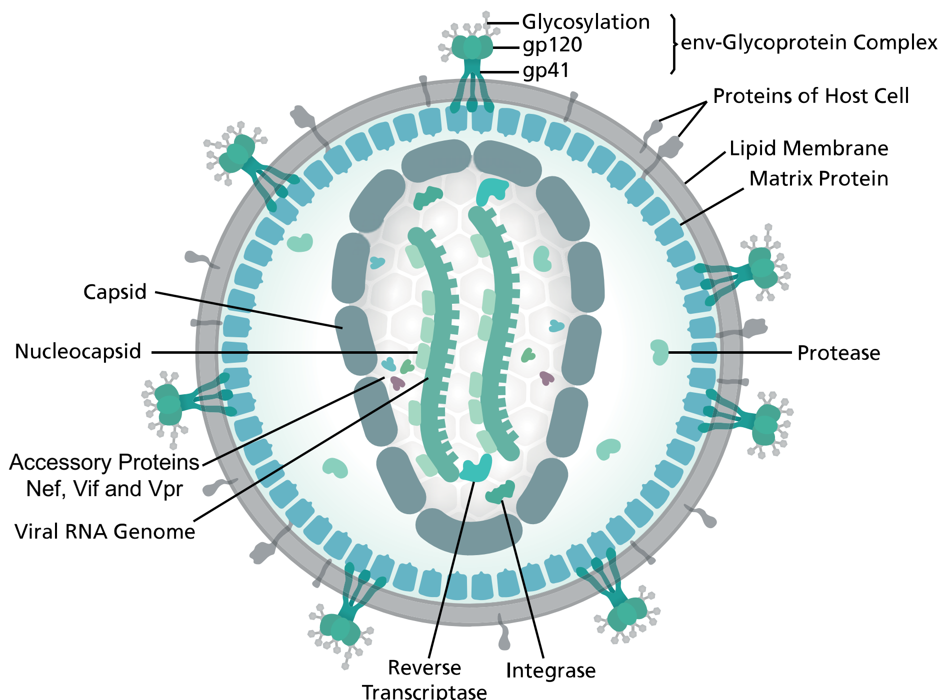


Figure 2. Schematic representation of HIV-1 structure showing the RNA genome and the viral proteins. Adapted from Splettstoesser T. (20).

Both RNA chains are strongly joined with p6 and p7 proteins, forming the NC, which is wrapped by 1,200-2,500 units of p24 viral protein, shaping a conical capsid (21,22). This CA also contains the reverse transcriptase and integrase en-

zymes, which are required for the early steps of the viral replication cycle, and is covered by approximately 2,000 copies of p17 (MA), which confers integrity to the viral particle and the icosahedral shape of the virus. The lipid bilayer is composed of lipids and proteins – including the Major Histocompatibility Complex (MHC) molecules - from the host cell membrane. It also contains the envelope viral proteins, which are organized in trimers of the glycoprotein gp120 and trimers of the glycoprotein gp41 that form the viral spikes (23). Additionally, the viral particle also contains the regulatory/accessory proteins Nef, Vif and Vpr, but not Rev, Tat and Vpu (24).

1.1.3. HIV-1 replication cycle

The HIV-1 replication cycle can be divided into five main stages (Fig.3):

1. Virus entry. This stage includes the binding of the virus to the host cell membrane, the fusion of both membranes, and the uncoating or release of viral RNA and proteins into the cytoplasm. The binding requires the interaction of the HIV-1 gp120 with the CD4 receptor on the surface of the target cell (25). After this interaction, the gp120/gp41 spike complex changes its conformation and allows for gp120 to interact with the CCR5 or CXCR4 co-receptors. This binding causes a conformational change in gp41 that leads to the fusion between the virus and the host cell. Subsequently, the viral capsid is internalized and the viral RNA and proteins are released into the cytoplasm of the cell (26).
2. Reverse transcription. Immediately after the uncoating, the viral RNA genome is retrotranscribed into linear double-stranded DNA (dsDNA) by the viral Reverse Transcriptase (RT) enzyme (27).
3. Nuclear import and integration. Following the reverse transcription, the dsDNA interacts with the viral Integrase and other proteins, forming the pre-integration complex (PIC), which is responsible for the nuclear import of the dsDNA. In the nucleus, the dsDNA is mainly integrated in the host cell genome by the enzymatic activity of the Integrase, constituting a provirus. From this moment on, the host cell remains permanently and irreversibly infected. This integrated proviral DNA can be replicated, transcribed and translated as any other cellular gene during the cell division process, and

thus produce new viruses. Alternatively, it can remain latent, in a reversible state of non-productive infection (28). However, depending on the Integrase activity and the activation status of the target cell, the dsDNA may not get integrated but rather remain as unintegrated DNA in the forms of linear, 1-long terminal repeat (LTR), or 2-LTR (29).

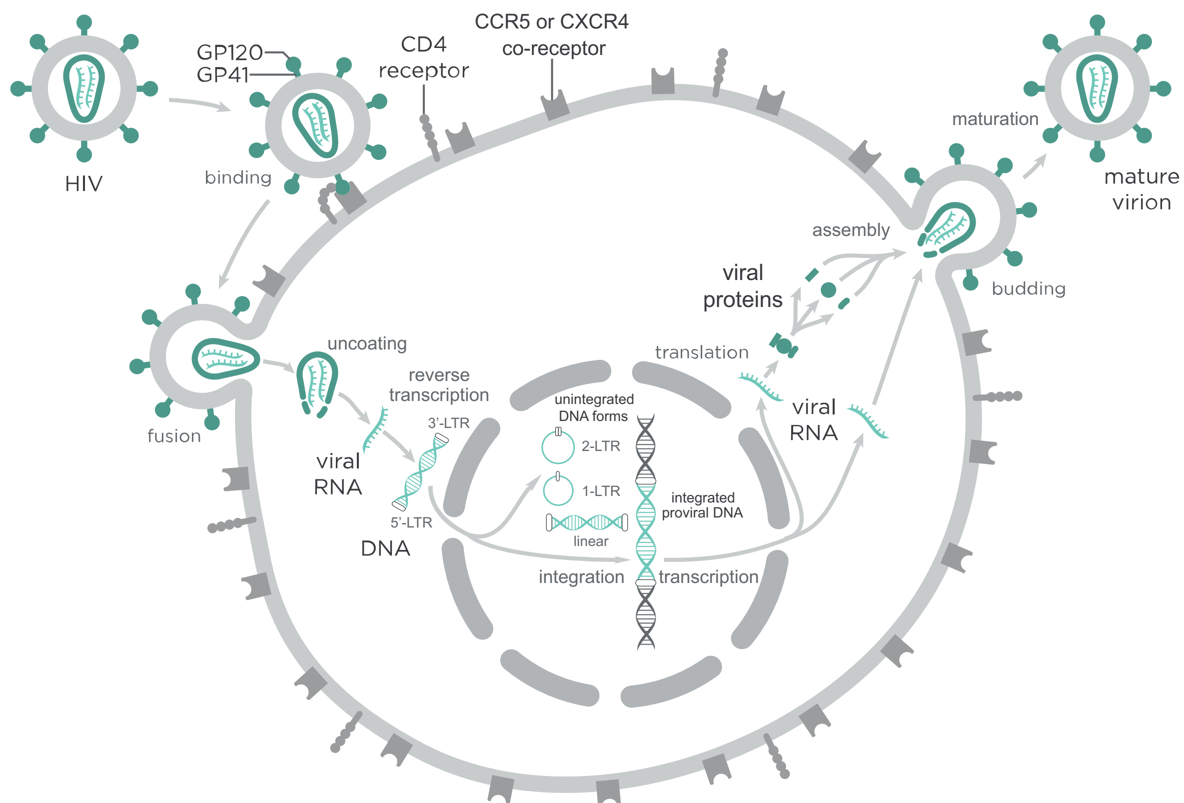


Figure 3. Schematic overview of the HIV-1 replication cycle. The figure illustrates the main steps in the HIV-1 life cycle divided into an early and a late phase. The early phase includes: the attachment of the virus particle to the CD4 receptor and co-receptors CCR5 or CXCR4; the fusion with the host cell membrane; the uncoating of the viral capsid; the release of the HIV-1 RNA genome and proteins into the cytoplasm; the reverse transcription of the viral RNA genome into a DNA duplex, which has terminal duplications known as long terminal repeats (LTRs); the translocation into the nucleus and the integration into the cell genome. In the nucleus, unintegrated viral DNA is found in both linear and circular forms. The unintegrated circular forms of viral DNA have either one or two LTRs. The linear unintegrated viral DNA is the precursor of integrated proviral DNA, which is a stable structure that remains indefinitely in the host-cell genome. After the integration, the late phase of the cycle starts, in which: the proviral DNA is transcribed to form new viral RNA, which subsequently is translated to form viral proteins; these proteins translocate to the cell surface to assemble in the cell membrane and form new viruses. Finally, the new viral particles bud off and are released as mature virions. Adapted from Splettstoesser T. (20).

4. Transcription and translation. After integration, the provirus is transcribed by the cellular polymerase II driven by viral promoters and enhancers in the LTR upstream of the transcription start site, and aided by the activity of the viral accessory proteins Tat and Rev, which are produced immediately after infection from multi-spliced (ms) RNA transcripts. Subsequently, HIV-1 tran-

scription increases and single-spliced mRNAs are produced, which encode for Env and the accessory proteins Vif, Vpr and Vpu. Later, full-length unspliced (us) transcripts, which act both as genomic RNA and as mRNA for the Gag-Pol polyprotein, are produced. All of these transcripts are exported to the cytoplasm, processed and translated (30,31).

5. Viral assembly, budding and maturation. Gag polyprotein and its proteolytic maturation products coordinate all of these stages. HIV-1 assembly occurs within specialized membrane microdomains. The virion packages all of the components required for infectivity: two copies of positive sense genomic RNA, tRNAs to prime the reverse transcription, Gag polyprotein, viral Protease, Reverse Transcriptase and Integrase enzymes, and the accessory proteins Nef, Vif and Vpr. The budding stage is mediated by the activity of the host endosomal sorting complexes required for transport (ESCRT) machinery. As the immature virion buds, viral Protease is activated and cleaves Gag into its constituent MA, CA, NC, and p6 proteins, converting the immature virion into its mature infectious form (24,32).

1.2. Natural course of HIV-1 infection

HIV-1 infects the cells of the immune system, preferentially the $CD4^+$ T lymphocytes, but this virus can also affect others cells such as monocytes, macrophages and dendritic cells (33,34).

The natural course of HIV –in the absence of antiretroviral therapy– consists of three different stages: the acute infection, the chronic phase and the Acquired Immunodeficiency Syndrome (AIDS). Overall, the HIV-1 cause a gradual decrease of the $CD4^+$ T cells, a chronic immune activation state and a subsequent exhaustion of the immune system, which leads to the manifestation of AIDS and unfortunately, in most cases, to the death of the patient (22) (Fig.4).

- Acute infection. Within 1-2 weeks of HIV-1 infection high levels of HIV-1 replication (10^5-10^7 HIV-1 RNA copies/ml plasma) and rapid loss of $CD4^+$ T cells occurs. Commonly, this phase is accompanied by clinical symptoms similar to mononucleosis, such as fever, lymphadenopathy, myalgia, rash,

etc. (35) and lasts from 1 to 6 weeks after infection, until anti-HIV-1 antibodies are detectable (36).

- Chronic phase. Following the induction of immune response to HIV-1, the viral load (VL), or level of HIV-1 in plasma, declines and is maintained stable in a value, which is known as the viral set point. This level of VL is strongly predictive of the clinical disease progression and the time when AIDS will appear (37–41). This stage usually lasts from 5 to 10 years and is characterized by a continuous decrease in the number of circulating $CD4^+$ T lymphocytes, and an overstimulation of the immune system, due to the persistent exposure to HIV-1 antigens and the altered cytokine environment (42).
- AIDS. This stage is characterized by levels of $CD4^+$ T-cell counts below 200 cells/μl, a dramatic increase of VL, and a complete failure of the immune system. The lowest $CD4^+$ T-cell count is known as $CD4^+$ nadir and in some patients decreases until 0 cells/μl. Thus, this situation allows for the establishment of opportunistic infections and HIV-1 associated cancers by which AIDS is defined, like Kaposi's sarcoma, pulmonary tuberculosis, bacterial pneumonia, esophageal candidiasis, etc. (43).

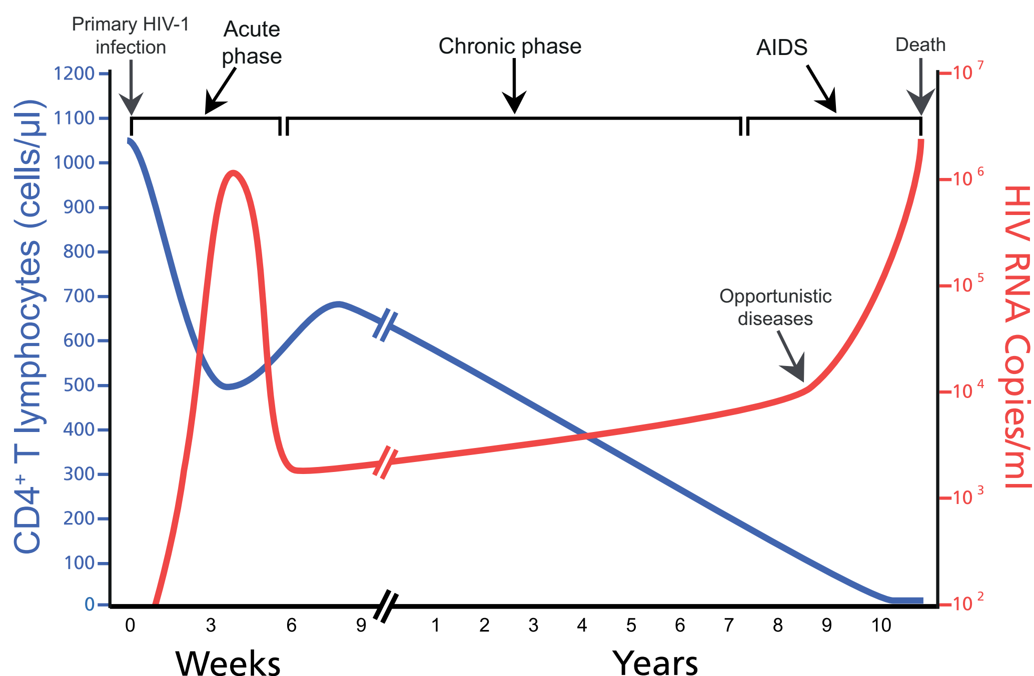


Figure 4. Schematic representation of the natural HIV-1 course of infection in absence of antiretroviral therapy. $CD4^+$ T lymphocyte count is shown in blue and HIV RNA copies/ml in red. Adapted from Splettstoesser T. (20).

1.3. Antiretroviral therapy and persistence of HIV-1 infection

In 1987, the first antiretroviral drug (azidothymidine, AZT) was approved and given as monotherapy (44). However, it was not until the mid-1990s that the treatment of HIV-1 infection was revolutionized by the development of new Reverse Transcriptase and Protease inhibitors, two of the three essential enzymes of HIV-1, and the introduction of drug regimens that combined these agents to enhance the efficacy of the therapy (45).

To date, more than 30 Food and Drug Administration (FDA)-approved drugs are available for the treatment of HIV-1 infection (46). These drugs are distributed into six distinct classes based on their molecular mechanism:

1. nucleoside-analog reverse transcriptase inhibitors (NRTIs),
2. non–nucleoside reverse transcriptase inhibitors (NNRTIs),
3. integrase inhibitors (InIs),
4. protease inhibitors (PIs),
5. fusion inhibitors, and
6. co-receptor antagonists.

Combination antiretroviral therapy (ART), administered in the chronic phase, efficiently suppresses viral replication and reduces the plasma HIV-1 viral load (VL) below the limits of detection of the most sensitive clinical assays (<50 RNA copies/ml), reconstituting the immune system (47–49). Importantly, combination ART using three antiretroviral agents directed against at least two distinct molecular targets is used to avoid the appearance of drug resistances, clinical drug failure and VL rebound (45,50). Moreover, combination ART has dramatically reduced morbidity and mortality associated with HIV-1 (51), and viral transmission (52). However, after treatment interruption, in most patients, HIV-1 VL rebounds and CD4 count drops, as in the acute phase. This is because ART inhibits viral replication but does not mediate the complete clearance of latently infected cells, whose proviruses are able to produce new mature virions. Therefore, although ART efficiently inhibits HIV-1 replication, it is not able to cure this infection due to the persistence of the viral DNA integrated into the cellular genome of the latently infected host cells.

Latent viral infection is a reversible nonproductive state of HIV-1 infection, characterized by the presence of infected cells that do not actively produce viral particles, but are able to do so upon stimulation, and constitute the HIV-1 reservoir (53). Combination ART reduces the HIV-1 reservoir during the first three years of therapy, but cannot eliminate all the latently infected cells that are the source of the HIV-1 VL rebound after ART discontinuation (Fig 5).

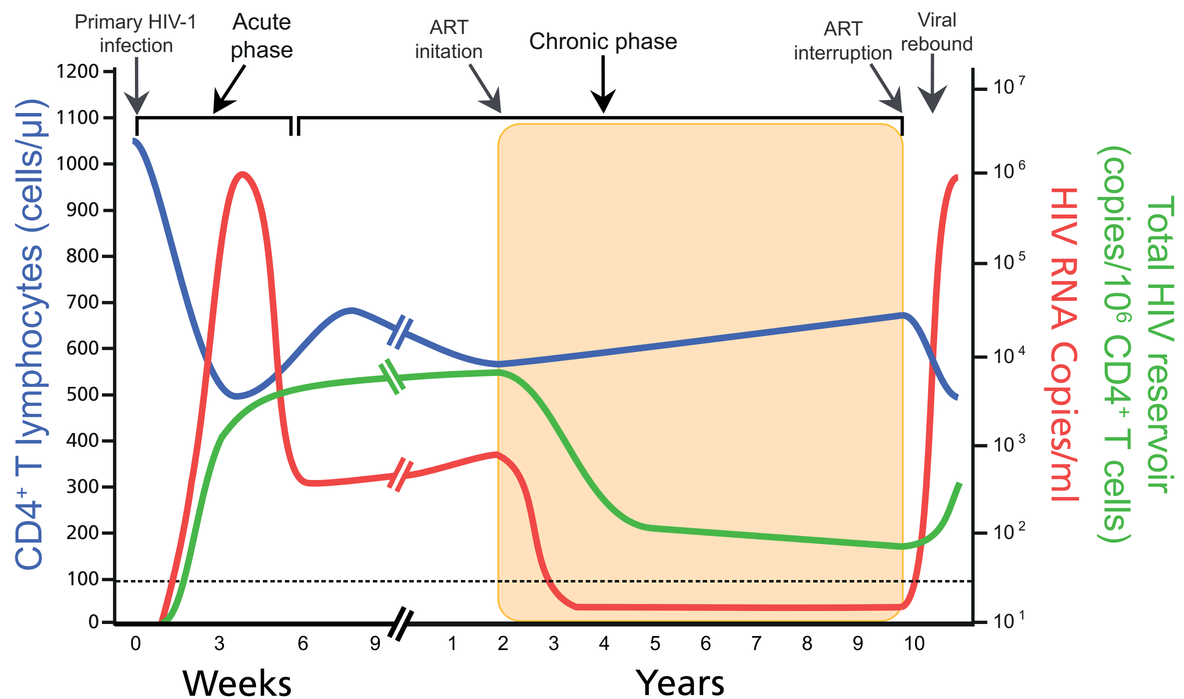


Figure 5. Schematic representation of the course of infection of HIV-1 on antiretroviral therapy. $CD4^+$ T lymphocytes count is shown in blue, HIV RNA copies/ml in red and HIV reservoir in green. Dot line represents the limit of detection (LOD) of the viral load (VL) by clinical assays (50 copies/ml). Adapted from Splettstoesser T. (20)

Several mechanisms contribute to HIV-1 persistence during ART, including antigenic and homeostatic proliferation of long-lived latently infected cells, immune dysfunction, and persistent residual viral replication in tissues and immune sanctuaries, where intracellular drug concentrations are lower and immune activation levels are higher (54,55) (explained in Figure 6).

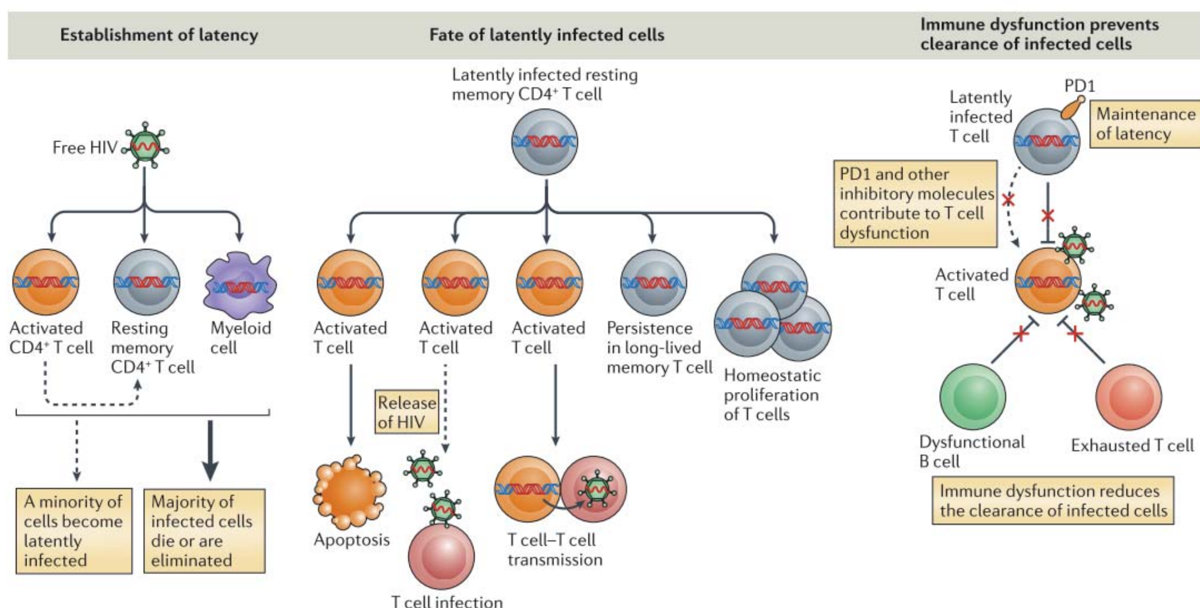


Figure 6. Mechanisms of HIV-1 persistence during antiretroviral therapy. The left panel illustrates how latent HIV infection can be established in T cell and myeloid cell reservoirs. The primary mechanism is probably infection of activated memory CD4$^+$ T cells. Most of these cells die, but a minority revert to a resting state. The center panel illustrates the fate of these now resting 'latently infected' memory CD4$^+$ T cells. These cells either die slowly, become a source of new infections, persist as long-lived cells or expand through homeostatic mechanisms. The right panel depicts some immune mechanisms that contribute to persistence. These include mechanisms that maintain cells in a resting state (for example, the upregulation of programmed cell death protein 1 (PD1)). Immune dysfunction during therapy probably reduces the efficient clearance of infected cells. Reproduced from Deeks S. *et al.* (56). Reprinted with permission from Nature Publishing Group.

The first HIV-1 reservoir described was located in the resting CD4$^+$ T cells (57,58). It is considered as the largest reservoir and the main barrier to achieving the cure of HIV-1 infection (58). Moreover, HIV-1 DNA has been detected in several peripheral CD4$^+$ T cell subsets, such as naïve (T_N), central memory (T_{CM}), transitional memory (T_{TM}), effector memory (T_{EM}), terminally differentiated (T_{TD}) cells and T stem cell memory (T_{SCM}), in different proportion (59,60). Specifically, Chomont *et al.* (61) identified that, among peripheral CD4$^+$ T cells, central memory (T_{CM}) and transitional memory (T_{TM}) are the major cellular reservoirs for HIV-1 in individuals treated with ART (61). In addition to the peripheral CD4$^+$ T-cell subsets, different cell types, such as macrophages, monocytes, microglia, astrocytes, T migratory memory (T_{MM}), T tissue resident memory (T_{RM}), and T follicular helper (T_{FH}), also contribute to the HIV-1 reservoir (62–64). Thus, the latently infected cells can be found in different lymphoid tissues, such as lymph nodes, gut-associated lymphoid tissue, central nervous system, etc., increasing the difficulty to completely eliminate the HIV-1 reservoir (64) (Fig. 7).

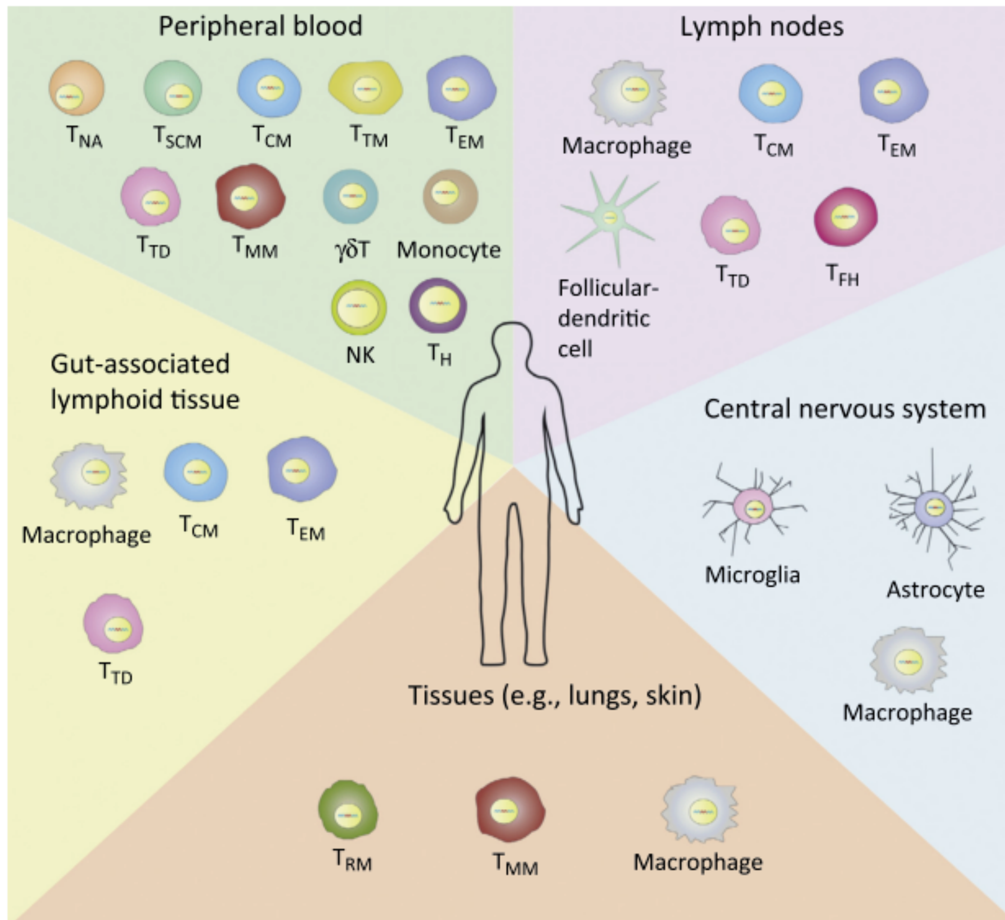


Figure 7. Schematic representation of the tissues and the specific tissue-cells contributing to the persistence of HIV-1 during effective ART. Reproduced from Barton K. *et al.* (64). Reprinted with permission from Elsevier.

1.4. Assays to quantify the HIV-1 reservoir

Research focused on the eradication of HIV-1 requires sensitive, precise, and practical assays to monitor the HIV-1 reservoir. Approximately, 1 in 10^5 –10^8 $CD4^+$ T cells harbors a replication-competent virus in most treated patients. The latent reservoir depends on the moment when ART was started after infection and where an individual's HIV-1 viral load set point was established (65).These low frequencies highlight the technical and practical challenges of measuring the HIV-1 reservoir. On the one hand, the technical challenges require the assay to be (i) sensitive enough to detect small numbers in a background of a large number of cells, (ii) specific so that a rare event is a true event, and (iii) capable of distinguishing between replication-competent and -incompetent proviruses. On the other hand, the practical challenges are to obtain enough cells to measure the rare events and to obtain enough rare events to measure a reduction of the HIV-1 reservoir (66).

Significant progress has been made in developing polymerase chain reaction (PCR)- and culture-based assays since the initial discovery of the latent reservoir, in the 1990s (67), to develop the assay which best estimates the HIV-1 reservoir. Nowadays, the most used assays are based on quantifying the total or integrated reservoir, the residual viral replication, the residual plasma viremia, the HIV-1 expression, the replication-competent reservoir and/or the inducible reservoir. The most sensitive, quickest, and easiest assays to measure the prevalence of HIV-1 infected cells are PCR-based assays, which quantify total, integrated or episomal HIV-1 DNA or HIV-1 RNA transcripts (66).

1.4.1. Total or integrated reservoir and residual replication – qPCR or ddPCR and Alu-PCR

In an HIV-1 infected cell, HIV-1 DNA can be found as integrated or unintegrated, as linear, 1-long terminal repeat (1-LTR), or 2-long terminal repeat (2-LTR) forms.

The most usual method to quantify HIV-1 DNA is quantitative real-time PCR (qPCR) for **total HIV-1 DNA**, which includes both integrated and unintegrated forms. Specifically, 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* or *pol* regions (68,69). However, this quantification assay requires a standard curve, constructed from known copy numbers of proviral DNA, to estimate the total HIV-1 copies and then these values are normalized to the total cell number using a cellular gene. This assay has been improved in different ways over time. On the one hand, a direct cell lysis protocol is commonly used, instead of column-based DNA extraction protocols, due to the fact that direct cell lysis improves the efficiency of nucleic acid recovery. On the other hand, qPCR limits assay accuracy at low copy numbers by exponentially amplifying noise and estimates but do not quantifies the HIV-1 DNA. Therefore, droplet digital PCR (ddPCR) is used as an alternative to qPCR, with improved accuracy, precision and direct quantification, using Poisson statistics (70). However, the quantification of total HIV-1 DNA overestimates the true HIV-1 reservoir, since this technique quantifies both replication-competent and defective proviruses (67,71).

Regarding **integrated HIV-1 DNA**, *Alu*-PCR is the most commonly used method (72–74). Briefly described, this protocol consists of a PCR with one primer binding *Alu* elements in the human genome and the second primer binding the HIV-1 *gag* or long terminal repeat (LTR) regions. This is then followed by a nested PCR with primers that amplify the HIV-1 LTR region. This assay also requires a standard curve to quantify the levels of integrated HIV-1 DNA, which has to include a mixture of cells with different HIV-1 integration sites at different distances from an *Alu* element. Integrated proviruses can also be quantified by physical separation of chromosomal DNA (75), a linker-primer PCR assay (76), or inverse PCR (77). HIV-1 integrated DNA strongly correlates with total HIV-1 DNA measured by ddPCR (68), indicating that most of the HIV-1 DNA in patients treated efficiently with ART is integrated. Thus, in HIV-1 ART-suppressed subjects, it is better to quantify total than integrated HIV-1 DNA, since the total proviral reservoir measurement is easier and more accurate.

As a result of failed integration, unintegrated forms of HIV-1 DNA, such as linear, 1-LTR or 2-LTR circles, can be detected in infected cells. These unintegrated forms are not considered part of the latent reservoir; however, based on their short-lived nature, **2-LTR circles** have been used as a measure of recent infection or residual viral replication during suppressive ART (78,79). Therefore, several assays have been developed to quantify these 2-LTR forms by both qPCR and ddPCR, using primers/probe sets that amplify the 2-LTR junction (68,78–80). Moreover, this assay has been improved regarding DNA sample extraction. For example, total nucleic acid extraction by direct cell lysis protocol is usually used, since it efficiently recovers more nucleic acid and is more sensitive than specific episomal column-extraction (81).

1.4.2. HIV-1 expression – RT-qPCR or RT-ddPCR

Most of the latently infected cells do not transcribe viral RNA; however, unspliced (us) and multi-spliced (ms) RNA transcripts have been detected in ART-treated individuals (82). usRNA corresponds to the first HIV-1 mRNA transcribed and msRNA represents the HIV-1 mRNA processed for being translated.

Cell-associated RNA (CA-RNA) can be measured from RNA extracts of different cell types, usually $CD4^+$ T lymphocytes. **usRNA**, the first HIV-1 RNA transcribed, can be measured by a seminested or a nested reverse transcription quantitative PCR (RT-qPCR) (83–85) or by RT-ddPCR (86,87), using primers/probe sets that amplify the viral genome regions of *5'LTR*, *gag* or *pol*. Otherwise, for **msRNA**, primers/probe sets that amplify the region containing the *Tat/Rev* exon-exon junction are used, which only appears in the msRNA, either with RT-qPCR or ddPCR platforms (83,84,88). Moreover, specific primers/probe sets, which amplify the polyadenylated tail in HIV-1 3'-LTR region are used to measure polyadenylated HIV-1 transcripts by RT-qPCR or RT-ddPCR (89,90).

HIV-1 expression can be used as a biomarker of viral persistence (91), because it reflects the ability to produce virus, although it does not directly relate to the production of replication-competent virions.

1.4.3. Residual plasma viremia – ultrasensitive viral load

Although ART efficiently suppresses plasma viremia below the clinical limit of detection (50 copies of HIV-1 RNA/ml plasma), HIV-1 RNA can still be detected at lower levels in the plasma of ART-treated patients, indicating residual viral replication (92).

In 2003, Palmer *et al.* (93) developed a highly sensitive assay, known as single-copy assay (SCA). This assay consists in the ultracentrifugation of up to 7 ml of plasma, followed by manually RNA extraction and a RT-qPCR assay with a standard control, and has been improved over time (94,95).

Alternatively, more automated assays have been developed to overcome the fact that SCA protocol is time-consuming. These assays consist in the ultracentrifugation of up to 7 ml of plasma, as for the SCA, but followed by an automated RNA extraction and RT-qPCR using the Abbott Real-Time HIV-1 assay (Abbott assay) or the Roche COBAS(®) AmpliPrep/COBAS(®) TaqMan(®) HIV-1 test, v. 2.0 (TaqMan(®) test v2.0) with standard controls (96–100).

Moreover, another approach, known as transcription-mediated amplification assay (TMA), has been developed. This assay quantifies the HIV-1 RNA copies/ml

based on the proportion of 0.5 ml aliquots of plasma providing RT-qPCR amplification, using Poisson statistics (101).

All of these assays require a large volume of plasma, use primers located in a conserved region of the viral genome and can detect down to 1 copy of HIV-1 RNA/ml plasma.

Overall, residual viremia quantification is a useful tool for measuring HIV-1 persistence in plasma. However, it does not directly determine the latent reservoir size or viral infectivity.

1.4.4. Replication-competent reservoir – qVOA and mVOA

The replication-competent reservoir represents the proviruses capable of transcribing, translating and producing new mature virions, and is traditionally quantified by culture-based assays.

The **quantitative Viral Outgrowth Assay** (qVOA) is considered the gold-standard technique for quantifying the frequency of $CD4^+$ T lymphocytes harboring replication-competent proviruses, although it underestimates the true HIV-1 reservoir (67,71). This technique underestimates the latently infected cells due to the fact that not all the replication-competent proviruses are reactivated and detected as infective viruses. Briefly, this assay consists in a limiting dilution cell culture, using total or resting $CD4^+$ T cells, which are first activated with phytohemagglutinin (PHA) and co-cultured with irradiated allogeneic PBMCs, and then with stimulated $CD4^+$ T cells from uninfected donors (102). After 2 weeks *in vitro*, viral production is measured by $p24^{Gag}$ ELISA assay. Improvements of this assay include using alternative cells for the co-culture, such as an established cell line (103) or CD8-depleted PBMCs from three uninfected donors activated with three different stimulus (known as 3×3 cells) (104,105), with the aim of achieving the maximum reactivation of the replication-competent reservoir.

The **murine Viral Outgrowth Assay** (mVOA) has been recently developed to detect replication-competent virus (106). Briefly, in this assay, cells or tissues from ART-suppressed HIV-1 infected subjects are transferred as xenografts into humanized immunocompromised mice, and the viral load in the plasma of these

mice is monitored by RT-qPCR. Although, mVOA is not quantitative, it is more sensitive than standard qVOA performed in parallel with samples from the same individuals. Therefore, this method may be useful when *ex vivo* assays cannot detect latent infection (66).

1.4.5. Inducible reservoir – TILDA

Several new assays based on quantifying the inducible HIV-1 reservoir have been developed, such as the Tat/rev-induced limiting dilution assay (TILDA) (107). In general, total or resting CD4^{+} T lymphocytes are activated, as for qVOA, and then cell-associated or supernatants HIV-1 RNA is measured by RT-qPCR. Thus, cell-associated RNA (CA-RNA) measures both transcriptionally-competent proviruses and defective transcripts, while HIV-1 RNA from supernatants reflects the capacity to reactivate proviruses that can release new virions. However, similar to HIV-1 total DNA determination, this assay overestimates the true HIV-1 reservoir, given that it can measure transcripts derived from defective provirus that can release new virions even if these are not replication-competent.

In summary, although the continuous development of techniques to accurately quantify the latently infected cells, more assays need to be developed in order to determine the true size of the reservoir (Fig. 8).

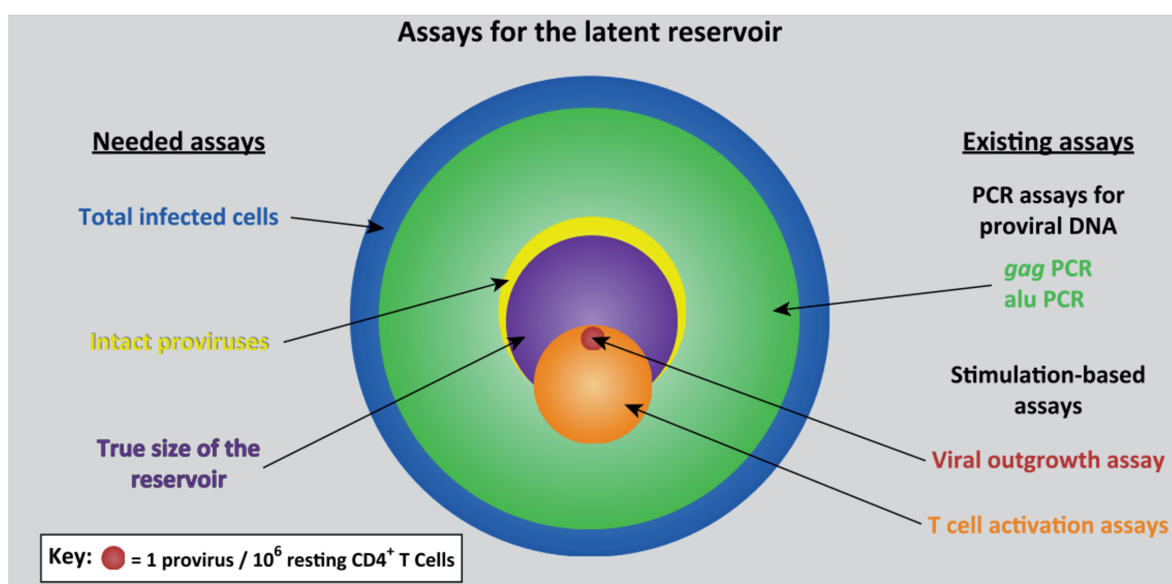


Figure 8. Venn diagram comparison of proviral populations measured by different methods for assessing the latent reservoir. Typical or estimated results from various PCR- and culture-based assays are shown relative to a prediction of the true latent reservoir size. The frequency of infected cells detected by the different assays is represented by the area of each circle. Reproduced from Bruner K. *et al.* (67). Reprinted with permission from Elsevier.

1.5. Therapeutic approaches for HIV-1 cure

Antiretroviral therapy (ART) inhibits HIV-1 replication, reduces rates of transmission and improves life expectancy. However, it does not cure the infection and it needs to be chronically administered to avoid viral rebound and drug resistance emergence. Therefore, alternative therapeutic strategies are being developed to find a cure for HIV-1 infection. These approaches are mainly focused on controlling or eliminating viral persistence. Thus, two definitions of HIV-1 cure have been described: the sterilizing or the functional cure. On the one hand, a **sterilizing cure** consists in the complete eradication of infectious forms of the virus from the body. On the other hand, a **functional cure** is defined as the control of HIV-1 replication after discontinuation of ART, known as post-treatment control (108).

Both types of cure are being pursued with multiple strategies, such as treatment optimization, immunotherapy, latency reactivation, therapeutic vaccination, and cellular or gene therapy.

1.5.1. Treatment optimization (intensification, switching and early-treatment)

One of the strategies to cure HIV-1 is based on ART optimization by adding more drugs to the current combination of three antiretroviral drugs (intensification). Substituting a drug of the current ART by another with lower side-effects, less daily doses or better tissue penetration (switching) or starting the ART as early as possible (early-treatment) are alternative approaches.

Regarding the **intensification of ART**, some studies have been performed in different cohorts of individuals with controversial results. Adding 1 or 2 drugs to the combination ART in ART-suppressed patients, naïve chronic or acute treated patients does not reduce the reservoir nor the residual plasma viremia (109,110). However, the addition of 1 or 2 drugs to chronic ART-suppressed or to naïve acute subjects results in a decrease in immune activation (78,79) and a faster HIV-1 RNA reduction in blood and seminal plasma (111), respectively. In addition, intensification with Integrase inhibitors resulted in a specific and transient increase in 2-LTRs forms, perturbing the residual viral replication despite not reducing HIV-1 reservoir size (78,79). Nevertheless, due to the debated results in ART intensified clinical trials, this strategy is not further considered as a potential HIV-1 cure.

With reference to the **switching of ART**, the same controversy as for intensification has been observed. One study demonstrated that switching to integrase inhibitors-based regimens does not impact the HIV-1 reservoir size (112), whereas a more recent study showed that those same regimens decrease the total HIV-1 reservoir (113). Therefore, switching drugs in ART-suppressed individuals is not further considered as a potential HIV-1 cure approach.

Respecting **early combination ART initiation**, some studies have demonstrated the importance of beginning therapy early after HIV-1 infection. For instance, the START trial showed less comorbidity and HIV-1 progression to AIDS (114), and the HPTN 052 trial also demonstrated a reduction in HIV-1 transmission (115) in subjects treated early. Moreover, other studies have also demonstrated that starting combination ART early on infection can result in a lower HIV-1 reservoir and immune activation levels, both in blood and tissue compartments, and better immune reconstitution (65,116–119). Additionally, two case reports published in 2011 and 2015, and the VISCONTI cohort study suggested that early treatment may result in more individuals becoming post-treatment controllers, which is the definition for the functional cure (120–122) (Fig. 9). Moreover, the case report of an intra-utero infected newborn treated 30 hours after birth, who was able to control viral replication after discontinuing ART at 18 months of age, suggested that the sterilizing cure might be achieved by an early initiation of the treatment (123). However, HIV-1 rebounded in that child after 27 months of ART interruption, demonstrating that the latent reservoir is established very quickly after infection (124,125).

Therefore, early combination ART initiation has to be considered as the best option for the optimization of treatment strategy to achieve a functional cure but not for a sterilizing cure. Nevertheless, more studies need to be performed to determine how early ART has to be started and for how long it has to be administered to get a remission of HIV-1 infection.

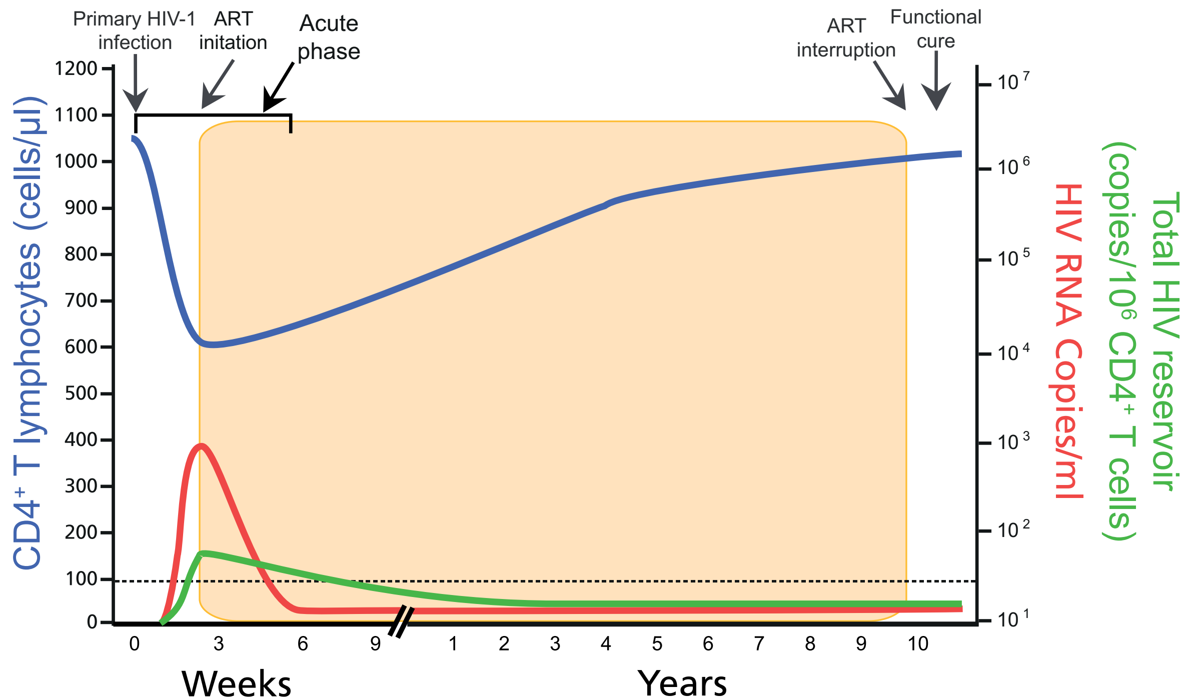


Figure 9. Schematic representation of the HIV-1 course of infection on early antiretroviral therapy. $CD4^{+}$ T lymphocytes count is shown in blue, HIV RNA copies/ml in red and HIV reservoir in green. Dot line represents the limit of detection (LOD) of the viral load (VL) and the HIV-1 reservoir. Adapted from Splettstoesser T. (20).

1.5.2. Immunotherapy

During HIV-1 infection the immune system is compromised because $CD4^{+}$ T lymphocytes are the main target of the virus, immune activation is not completely reduced by ART, and the continuous exposure to HIV-1 antigens causes the exhaustion of the immune system (126,127). Therefore, in this situation, it is very difficult to generate specific immune responses against HIV-1 to efficiently eliminate the productively infected cells.

Thus, immunotherapy has been proposed as a strategy to achieve an HIV-1 cure. This approach is based on the administration of cytokines or antibodies to restore the hyperactivated and exhausted immune system. Hence, this approach specifically targets the inflammation and cell exhaustion caused by the HIV-1 infection.

On the one hand, some **cytokines**, such as the interleukins (IL) -2, -7, -15 and -21, have been proposed as a potential immune-based therapy given that they seem to be able to increase $CD4^{+}$ and $CD8^{+}$ T-cell counts (128–134), which may restore the immune responses. However, these cytokine-based agents are of un-

certain benefit in HIV-infected patients since high levels of certain cytokines may participate in tissue inflammation and immune cell activation, which in turn could increase the number of potential HIV-1 target cells susceptible to infection (135).

On the other hand, **antibodies targeting exhaustion markers**, such as PD-1, PD-L1, or CTLA-4, have been developed to recover the functional status of the immune system. A recent study (136) suggests that blocking PD-1 triggers HIV-1 replication and might therefore reactivate latent viruses, allowing the body to eliminate these productively infected cells by cell death and/or clearance by HIV-specific immune responses (137). Curiously, PD-1 has also recently emerged as a potential target to facilitate HIV-1 eradication because $CD4^+$ T cells expressing high levels of PD-1 are latently infected in a higher proportion than PD-1 low cells (61). Moreover, another

Thus, in combination with ART, certain cytokines may be able to restore the immune system without increasing the HIV-1 reservoir size by homeostatic or antigen-dependent proliferation, and antibodies targeting exhaustion markers could also be a feasible strategy to increase and improve HIV-1-specific immune responses and sufficiently reduce the HIV-1 reservoir to achieve a functional or a sterilizing cure of this infection.

1.5.3. Latency reactivation

The major barrier to cure HIV-1 infection is the ability of HIV-1 to establish latent reservoirs of reactivation-competent proviruses. Thus, a third method proposed for the cure of HIV-1 consists in treating patients with latency-reversing agents (LRAs) in combination with ART. The aim of this strategy is to reactivate all the HIV-1 latent proviruses, which constitute the HIV-1 reservoir, to allow the immune system to recognize and completely eliminate the infected cells. This strategy is thus also known as **“shock and kill”** (53), in which the “shock” corresponds to the reactivation of the latent proviruses, and the “kill” to the clearance of the reactivated cells by the host immune system or by viral cytopathic effect. Thus, administration of LRAs needs to be done in combination with ART to avoid new cycles of replication by the reactivated proviruses.

Multiple classes of small-molecule LRAs have been identified to potentially reactivate the latently infected cells (138). These LRAs are involved in gene transcription regulation. Therefore, reactivating the latent virus without inducing global T-cell activation requires understanding the mechanisms that perpetuate latency.

During latency, **histone deacetylases** (HDACs) are recruited to HIV-1 LTR by the host transcription factors, and hypoacetylated nucleosome-1 (Nuc-1) prevents proviral transcription. In addition to acetylation, the histone H3 methylation by **histone methyltransferases** (HMTs), and methylation of CpGs islands flanking the HIV-1 transcription start site hypermethylation by **DNA methyltransferases** (DNMTs) are associated with transcriptional silencing of the provirus. Moreover, cytoplasmic sequestration of **host transcriptional factors** such as protein kinase C (PKC), nuclear factor κB (NF-κB) and positive transcription elongation factor b (P-TEFb), can also participate in the induction of latency (138). Thus, inhibitors of HDACs, HMTs and DNMTs, and inductors of PKC, NF-κB and P-TEFb pathways can be used as LRAs to purge the HIV-1 reservoir (Fig. 10).

However, none of the LRA tested to date has produced a substantial decrease in the size of the latent reservoir. This fact may be explained because this “shock and kill” strategy needs the induction of HIV-1 specific immune responses to efficiently recognize and eliminate the infected cells (139). Therefore, the shock step of this approach, performed by the administration of LRAs, ought to be combined with some sort of therapeutic vaccination, which induces specific HIV-1 responses, improving the killing step.

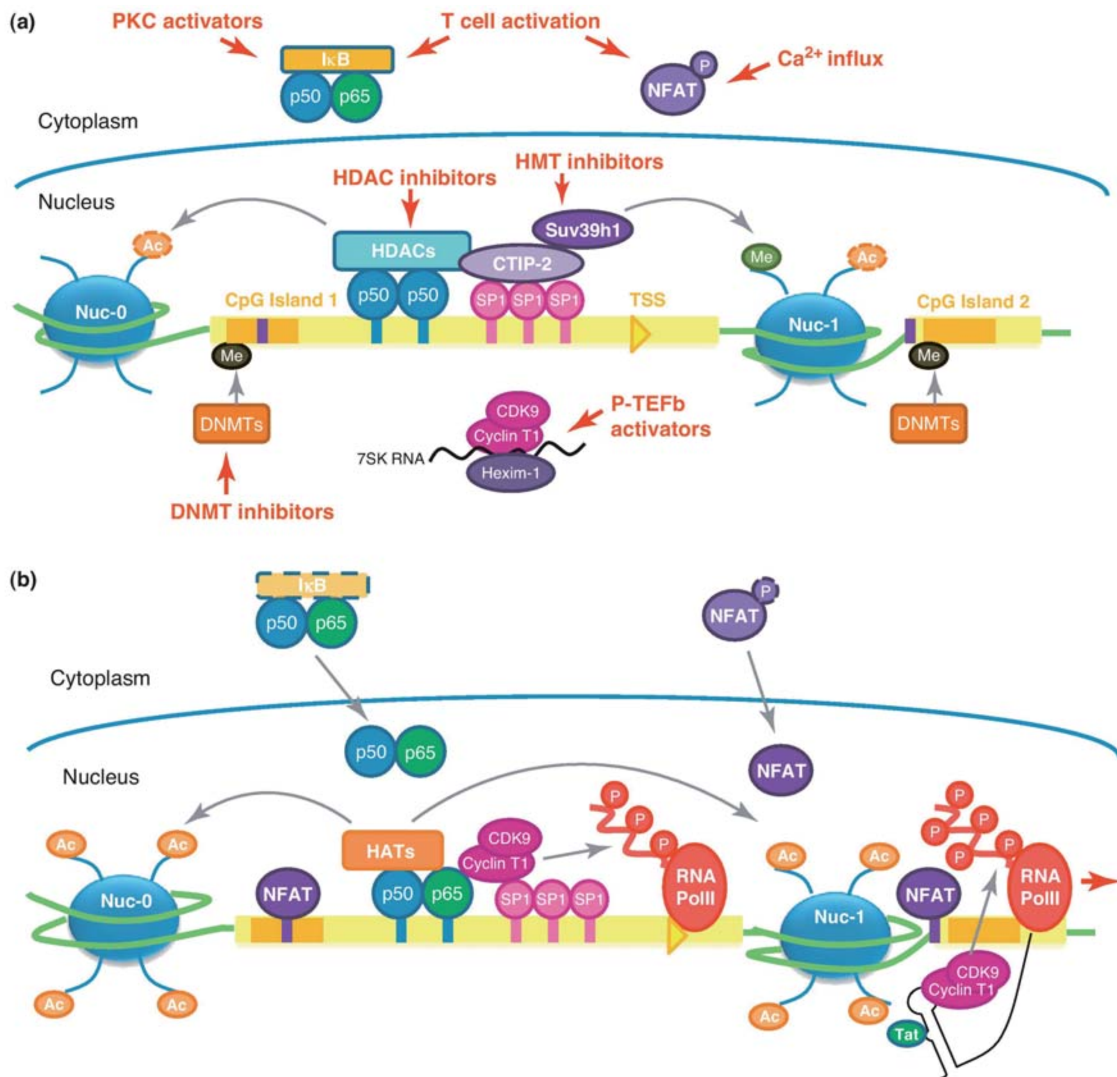


Figure 10. Schematic representation of the maintenance of HIV-1 latency and reactivation of latent HIV-1 provirus. The HIV-1 DNA is depicted as a green ribbon, with the two nucleosomes, Nuc-0 and Nuc-1, depicted as blue spheres. Parts of the HIV-1 5′LTR that contain binding sites for major transcription factors (boxes in purple, blue and pink) and the transcriptional start site (TSS, yellow triangle) are highlighted in light yellow. (a) The maintenance of HIV-1 latency and potential targets for reactivation. In latent infection, HDACs and HMTs are recruited to HIV-1 LTR, resulting in histone deacetylation and methylation on Nuc-0 and Nuc-1, which leads to a restrictive environment for transcription initiation. DNA methyltransferases could introduce DNA methylation on the CpG islands, which could further silence transcription. Most host transcriptional factors are sequestered in the cytoplasm in resting cells. The inactive NF-κB p50 homodimer binds to the NF-κB site at HIV-1 LTR, whereas the active form, a p65/p50 heterodimer, is bound by IκB in the cytoplasm. NFAT is in its phosphorylated inactive form. P-TEFb is restricted in a transcriptionally inactive complex with Hexim-1 and 7SK snRNA. To overcome these obstacles to transcription of the HIV-1 provirus agents targeting these restrictive steps have been explored for the reactivation of latent HIV-1 (red arrows). (b) The active transcription of HIV-1 provirus. Upon cellular activation, IκB is phosphorylated and degraded, releasing p65/p50 which translocates into the nucleus and binds to NF-κB sites on HIV-1 LTR. HATs are recruited to LTR, and acetylation disrupts histone–DNA binding, facilitating the recruitment of transcription factors and complexes. Cellular activation also results in the release of active P-TEFb which phosphorylates the C-terminal domain of RNA polymerase II (RNA Pol II), stimulating transcriptional elongation. After the stem-loop structure of TAR is transcribed, HIV-1 Tat efficiently recruits active P-TEFb to TAR, further stimulating the elongation of HIV-1 transcripts. Reproduced form Xing S. and Siliciano R (138). Reprinted with permission from Elsevier.

1.5.4. Therapeutic vaccination

Anti-viral T-cell and B-cell responses play a crucial role in suppressing HIV-1 during chronic infection (140). Therefore, active and passive immunizations strategies are currently explored to enable a functional cure by inducing control over HIV-1 replication.

Therapeutic vaccines for HIV-1 infection aim at eliciting anti-viral responses by $CD8^+$ T cells or cytotoxic T lymphocytes (CTLs), $CD4^+$ T cells, and B cells, which specifically generate neutralizing antibodies, in order for these immune responses to control viral replication (141–144). Moreover, these vaccination strategies have to generate broad responses, as HIV-1 mutates very rapidly to escape immune pressure (145). Indeed, the predominance of CTL-resistant viruses in the reservoir is a major barrier to viral eradication. Therefore, most of the immunogens used for therapeutic HIV-1 vaccines are designed upon conserved epitopes and elements of the viral genome that have an impact on the viral capacity of replication, with the purpose of complicating the immune escape of the virus (146).

Active immunization implies that, in addition to the development of an immunogen able to induce broad immune responses, this immunogen is combined with an adjuvant and a vector that direct and enhance the generated immune responses. The immunogen can be formulated in different presentations including or not a vector. In ascending order, beginning with the safest and less immunogenic, there are: naked DNA, peptides, soluble protein, viral-like particles, replication-deficient viral vectors, replicating viral vectors, killed whole viruses or live attenuated viruses (Fig. 11) (147).

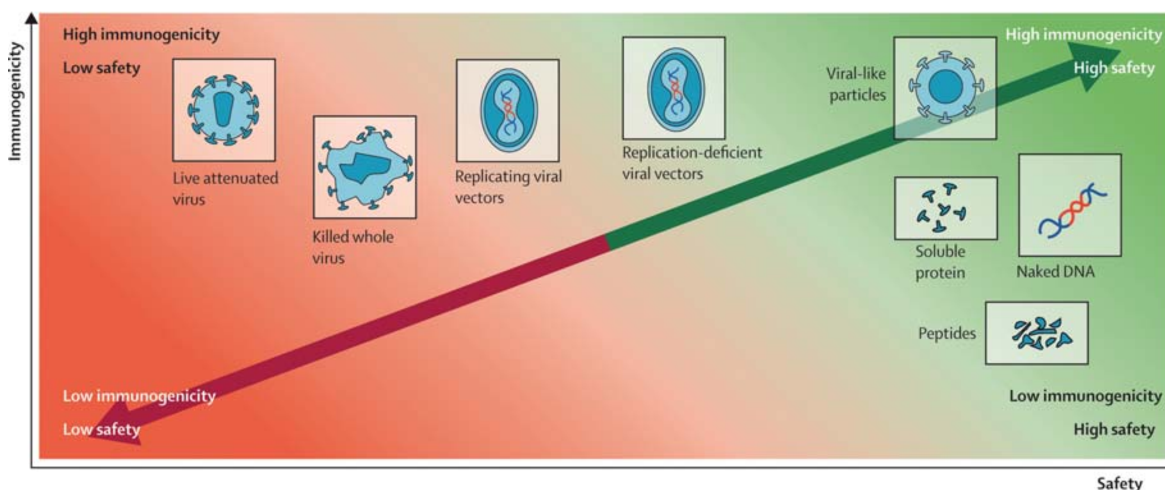


Figure 11. Schematic representation of different HIV-1 vaccine strategies. HIV-1 strategies have been distributed according to their immunogenicity and safety. The representation on the two axes are based on an arbitrary comparative scale. Reproduced from Ross AL. *et al.* (147). Reprinted with permission with Elsevier.

The most broadly used vaccine strategy to elicit **CTLs responses** is the combination of conserved epitopes and elements with a replication-deficient viral vector, since these vectors are safe and immunogenic enough. Moreover, some studies demonstrated that vaccines using these viral vectors increase CTL immune responses (148–151). Some studies performed in rhesus macaques infected with simian immunodeficiency virus (SIV), which were treated with an immunogen based on conserved SIV epitopes combined with the replicating viral vector derived from rhesus cytomegalovirus (RhCMV), showed that the combination of this viral vector with conserved epitopes mediates a progressive clearance of SIV infection (152,153).

Concerning **B-cell responses**, initially, HIV-1 vaccines focused on envelope-based vaccines to elicit the production of broadly neutralizing antibodies (bNAbs). However, the current use of bNAbs in HIV-1 infection differs from these traditional vaccination approaches. Specifically, this strategy employs **passive immunization** using the direct injection of a large quantity of selected bNAbs, instead of the immunogen that can elicit bNAbs secretion (53). A recent phase I clinical trial, where bNAb 3BNC117 was injected in HIV-1 infected patients not undergoing ART, showed that this bNAb was well tolerated and that, more importantly, it was able to reduce patient's viral load without the use of ART (154). Hence, infusion of bNAbs may be a safe and efficient method for delaying viral rebound after ART discontinuation. Moreover, bNAbs can both block new HIV-1 infection and target productively infected cells for destruction by antibody-dependent cell-mediated

cytotoxicity, in contrast to antiretroviral drugs that only block new infections (155). Thus, this passive vaccination strategy might be an effective approach to achieving a functional or even a sterilizing cure.

1.5.5. Cellular or gene therapy

Cellular or gene therapy strategies aim at curing HIV-1 infection by the transplantation of virus-resistant hematopoietic stem and progenitors cells, and, therefore, generating an HIV-resistant immune system. These virus-resistant cells can be derived from a naturally resistant healthy donor, by hematopoietic stem cell transplantation (HSCT), or by direct genome editing of the cells of the HIV-1 infected individual treated.

These approaches are mainly focused on the role of CCR5 as a co-receptor for HIV-1 entry in $CCR5^+$ $CD4^+$ T-cells, based on the fact that homozygous carriers of a 32-bp deletion in the *CCR5* gene (*CCR5*-Δ32/Δ32) are naturally resistant to the strain of HIV-1 that uses CCR5 to bind the host cell (R5 tropic) (156,157).

HSCT has achieved the only confirmed case of HIV-1 infection cure, commonly known as the “Berlin patient”. This was accomplished by Hütter *et al.* (158) with HSCT using peripheral progenitors cells from an HLA-matched adult donor who was homozygous for the 32-bp deletion in *ccr5*. The Berlin patient has remained without any evidence of HIV-1 infection for more than 8 years after discontinuation of ART, suggesting that he has been cured. This case encouraged enthusiasm for the development of an approach that achieves the cure of both HIV-1 infection and leukemia or lymphoma. Nevertheless, the case of the “Boston patients”, who suffered from HIV-1 infection and lymphoma and received a wild-type-*CCR5* allogeneic HSCT, showed disappointing results. Both patients experimented viral load rebound after 12 and 32 weeks of ART discontinuation, although the levels of plasma viral RNA, qVOA and cell-associated proviral DNA in peripheral blood mononuclear cells and tissues remained undetectable following the transplantation (159). Thus, the HSCT using *CCR5*-Δ32/Δ32 hematopoietic stem cells is a more promising approach than a wild-type-*CCR5* HSCT to achieve a sterilizing cure of HIV-1 infection. However, identifying an adult HLA-matched *CCR5*-Δ32/Δ32 donor is not readily feasible in part because of the low prevalence of the homozygous

variant allele Δ32 (about 1% in northern European descents) (160,161), and also because of the need for a very close HLA match between donor and patient (162). In contrast, cord blood *CCR5*-Δ32/Δ32 units provide a major advantage for HSCT because this transplant requires much less stringent HLA matching between donor and patient (163). Hence, HSCT using cord blood *CCR5* homozygous Δ32 can be more feasible than *CCR5*-Δ32/Δ32 cells from an HLA-matched adult donor, and therefore better for achieving an HIV-1 cure.

Considering the **genome editing** of patients' cells, different approaches have been developed. Specific genes can be directly modified using zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), or the clustered interspaced short palindromic repeats/CRISPR-associated protein-9 nuclease (CRISPR/Cas9) technology (53). All of these techniques can target a specific sequence of DNA and produce nucleotide editing (164). Multiple approaches of gene therapy for HIV-1 cure strategies are currently being evaluated.

The first approach studied has been the modification of the *CCR5* gene in $CD4^+$ T lymphocytes followed by the reinfusion of the modified cells, with the aim of blocking the binding of HIV-1 to the infused host cells. However, the cells reinfused represent a minority of the total $CD4^+$ T cells of the patient, and after ART interruption HIV-1 VL rebounds (165). Therefore, a promising approach to this technique is to modify hematopoietic stem cells instead, which would then replenish the CD4+ T lymphocyte pool with HIV-1 resistant cells.

Another approach consists in directly targeting the HIV-1 provirus. Some recent studies, performed in the model of the latent HIV-1 cell line J-lat and in animal models, have demonstrated that CRISPR/Cas9 technology can be used to mutate part of the provirus or even to cleave it entirely out of the cell genome (166–168).Thus, this strategy may be used to inactivate latent proviruses in HIV-1 infected patients with the aim of reducing the HIV-1 reservoir size and therefore achieving a functional or a sterilizing cure. Nonetheless, this treatment requires an effective delivery of the editing enzymes into every infected cell *in vivo*, which would be quite challenging to achieve and would require further development of this technology.

An alternative approach aims to enhance the immune response of HIV-infected cells to potentially control infection without the need for ART (53). This includes the introduction of HIV-specific T cell receptor (TCR) genes to $CD8^+$ T lymphocytes to allow for better recognition of infected cells (Chimeric Antigen Receptors), generating a more effective control of viral replication and likely inducing a functional cure (169).

In summary, genome editing can be considered as a promising research field to aid in the search of an HIV-1 cure.

CHAPTER 2. HYPOTHESIS AND AIMS

Despite the fact that current combination antiretroviral therapy (ART) effectively suppresses viral replication, HIV-1 remains an incurable viral infection that generates 2 million new infections per year (18). Moreover, ART discontinuation generally results in a quick viral rebound due to viral replication from the latent reservoir, which is established very early in the first stages of the infection (124,125). Thus, many approaches aimed at curing HIV-1 infection have been investigated and developed to find one (or a combination of them) that achieves a complete eradication of HIV-1 reservoirs (sterilizing cure) or a control of the viral replication in the absence of ART (functional cure). The current strategies investigated are treatment optimization, immunotherapy, latency reactivation, therapeutic vaccination and cellular or gene therapy.

Hence, the **hypothesis** of this thesis is that one or a combination of several of these strategies may impact the HIV-1 reservoir and, thus, achieve a remission of HIV-1 infection in patients receiving ART.

The **global aim** is to evaluate, compare and determine which HIV-1 cure approach (immunotherapy, latency reactivation, therapeutic vaccination or cellular therapy) has the highest probability to achieve a sterilizing or a functional cure.

The **specific aims** for each cure strategy analyzed are:

1. Immunotherapy:

Aim 1.1. To determine whether short-term treatment with interferon-α combined with ribavirin could affect HIV-1 transcription, the residual plasma viremia, the total HIV-1 reservoir, the replication-competent reservoir, and the residual viral replication, in ART suppressed HIV/HCV−coinfected subjects.

Aim 1.2. To identify a short-term mechanism by which interferon-α could impact the HIV-1 reservoir.

2. Latency reactivation:

Aim 2.1. To evaluate whether the use of lithium carbonate for the potential treatment of HIV-1-associated neurocognitive disorders could impact HIV-1 transcription, residual plasma viremia and total HIV-1 reservoir, in ART suppressed patients.

Aim 2.2. To determine whether treatment with lithium carbonate could act as a latency reversing agent (LRA) *in vivo*.

3. Therapeutic vaccination:

Aim 3.1. To evaluate whether therapeutic vaccination with the MVA.HIVconsv immunogen could re-direct T cell responses towards viral epitopes that are vulnerable and typically sub-dominant, in ART suppressed HIV-infected patients.

Aim 3.2. To analyze whether the specific elicited HIV-1 immune responses may have an impact on the total HIV-1 reservoir and the residual plasma viremia.

4. Cellular therapy:

Aim 4.1. To evaluate whether allogeneic hematopoietic stem cell transplantation (HSCT) using *CCR5* Δ32 homozygous cord blood (CB) cells could be a feasible therapeutic strategy to cure HIV-1 infection.

Aim 4.2. To determine whether an allogeneic HSCT using a compatible *CCR5* Δ32 homozygous CB could eradicate the HIV-1 in a patient with HIV-1 infection and a diffuse large B-cell lymphoma (DLBCL).

CHAPTER 3. CLINICAL TRIAL RESULTS

CHAPTER 3A. Immunotherapy

This chapter corresponds to the manuscript:

Short-term treatment with interferon alpha diminishes expression of HIV-1 and reduces CD4⁺ T-cell activation in patients coinfected with HIV and hepatitis C virus and receiving antiretroviral therapy

Sara Morón-López, Elisabet Gómez-Mora, Maria Salgado, Dan Ouchi, Maria C. Puertas, Víctor Urrea, Jordi Navarro, Antoni Jou, Mercedes Pérez, Cristina Tural, Bonaventura Clotet, Luis J. Montaner, Julià Blanco, Manuel Crespo, Javier Martinez-Picado

S.M-L. first author

JID, 2016

3A.0. Presentation

This chapter evaluates the treatment with the cytokine interferon-α (IFNα) as an immunotherapeutic strategy to cure HIV-1 infection, due to its antiviral action and its potential impact on the HIV-1 reservoir.

3A.1. Introduction

Current combination antiretroviral therapy (ART) quickly and persistently suppresses viral replication, thus increasing the life expectancy of HIV-1-infected individuals to almost that of non-infected individuals (170). However, long-term combination ART-associated issues, including poor adherence, the potential emergence of resistance, and cumulative toxicities, as well as economic and logistical considerations, have spurred interest in identifying new medical interventions aimed at achieving viral remission. Complementing ART with immunotherapy and chemotherapy has been proposed as a means of reactivating latent virus, reducing the size of the viral reservoir, and, ultimately achieving a functional cure (56,171,172).

Interferon-α (IFNα) is a type I interferon with potent antiviral activity against a large variety of infections. Until recently, IFNα combined with ribavirin (RBV) was the standard treatment against infection by the hepatitis C virus (HCV). IFNα-RBV still represents the backbone of some regimens in combination with new direct anti-HCV drugs (173).

Multiple studies of untreated HIV-1-infected patients confirmed that IFNα can reduce plasma viremia by 0.5–1.0 $\log_{10}$ in both HIV-monoinfected patients (174–177) and HIV/HCV-coinfected patients (178). Such antiviral activity in patients with actively replicating HIV-1 is likely related to the IFN-mediated up-regulation of a group of host genes that can restrict viral replication at various stages of the replication cycle (177,179).

Azzoni et al. (180) recently reported that long-term treatment (at least 7 months) with pegylated IFNα-2a might be able to control HIV-1 replication in a significant number of patients following discontinuation of ART. The authors observed a reduction in proviral integrated HIV-1 genomes in peripheral blood mononuclear cells (PBMCs) among patients who maintained viral suppression after discontinuation of ART. These findings were supported by the results of 2 studies of long-term treated HIV/HCV–coinfected patients (181,182). Taken together, the data suggest that long-term IFNα might reduce the size of the viral reservoir. However, the short-term mechanism by which IFNα diminishes the viral reservoir and its effects on activation and persistent HIV-1 expression remain unknown. Such knowledge

might be clinically relevant, as it could pave the way for short-term and long-term interventions aimed at reducing viral replication and remission.

In a cohort of HIV/HCV−coinfected patients on suppressive ART, we analyzed variations in cell-associated HIV-1 RNA, replication-competent virus, and expression of specific IFN-stimulated genes (ISG) encoding HIV-1 restriction factors and the protein Siglec-1 in peripheral $CD4^+$ T cells. We also assessed changes in residual plasma viremia, total HIV-1 reservoir, 2-LTR circles, distribution of peripheral T-cell subsets, and expression of these subsets in markers of activation and exhaustion during treatment with IFNα-RBV. Our objective was to identify a short-term mechanism by which IFNα could impact the HIV-1 reservoir.

3A.2. Material and Methods

3A.2.1 Patients

We performed an open, prospective, non-randomized cohort study at 2 university hospitals in Barcelona, Spain. The study population comprised 21 HIV/HCV–coinfected patients on suppressive ART for at least 6 months: 10 treated with pegylated IFNα-2a (180 µg/wk; Pegasys®, Roche) and RBV (800-1200 mg bid) (IFN group) and 11 patients whose anti-HCV treatment was deferred (control group). The participants gave their written informed consent, and the institutional review boards and the Catalonian Health Department (code: FHU-INT-2013-01) approved the protocol.

We collected total blood samples (80 ml) at study days 0 and 28. Peripheral blood mononuclear cells (PBMCs) were used for flow cytometry analysis. For the remaining analyses, $CD4^+$ T cells were isolated using negative immunomagnetic selection (Miltenyi).

3A.2.2. Peripheral Blood Mononuclear Cells (PBMCs) isolation and preservation

Blood samples from HIV-1 infected patients were processed in order to separate plasma from cell fraction. Plasma samples were stored at -80ºC and PBMCs were isolated from the cell fraction by a standard Ficoll-Hypaque (Lymphoprep, ATOM, AXIS-SHIELD PoC AS) density gradient centrifugation (390xg, 30 min.), aliquoted in cryo-vials (10-15 million PBMCs per vial), placed in a CoolCell® freezing container (BioCision) at -80ºC overnight, and stored in a liquid nitrogen until use (Fig. 12).

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; Miltenyi Biotec) using the "depletes" software program of autoMACS® Pro Separator (Miltenyi Biotec), following the manufacturer's instructions. 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). Purity was higher than 90% in all the samples (Fig. 13).

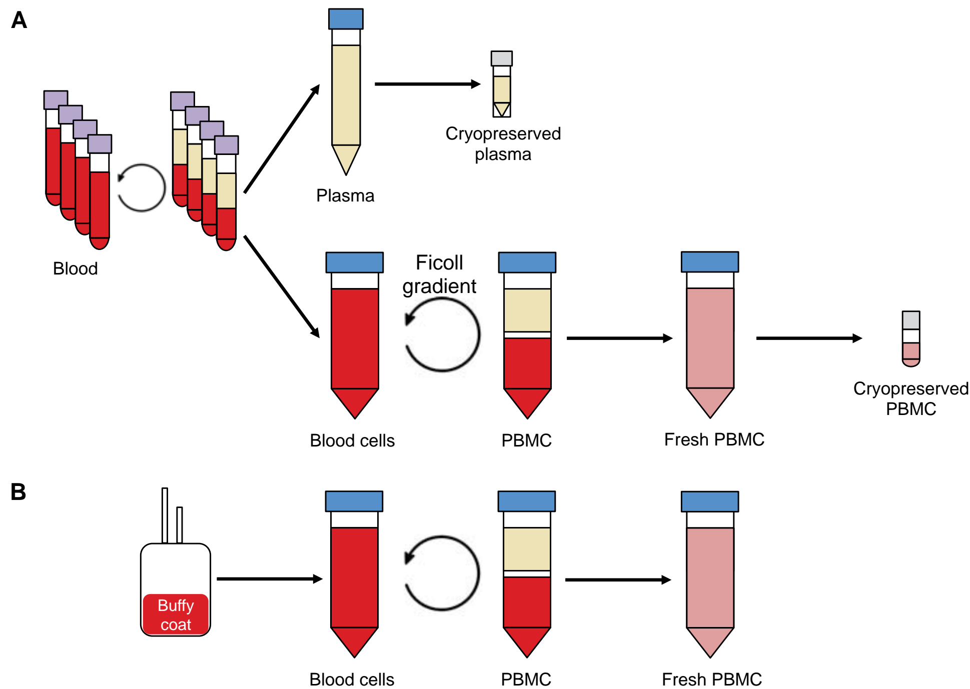


Figure 12. Schematic view of PBMCs isolation from **(A)** HIV-1 infected patients' blood samples and **(B)** buffy coats of HIV-1 negative donors

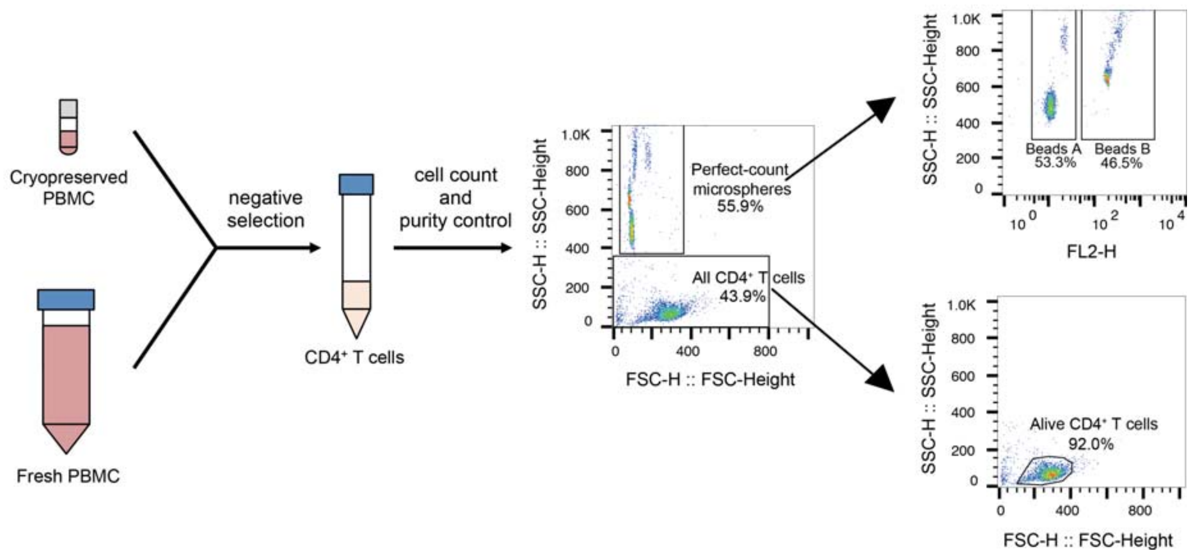


Figure 13. Schematic representation of the general procedure to obtain, count and check the purity of $CD4^+$ T cells isolated from the PBMCs of HIV-1 infected patients

3A.2.4. Cell-associated HIV-1 mRNA determination

To quantify cell-associated HIV-1 transcription, $CD4^+$ T cells (1×10^6) were purified from frozen PBMCs and 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 droplet digital polymerase chain reaction (RT-ddPCR) with the primers/probe sets located in the viral 5'LTR (73) and Gag (93) regions (Table 1), depending on the efficiency of detection in each patient due to mismatches or deletions in the viral sequences. For that reason, all samples were measured in parallel using both primers/probe sets to ensure effective and reliable proviral absolute quantification. The expression of the housekeeping gene TATA-binding protein (*TBP*) was quantified in parallel to normalize RNA input (183) (Table 1). Briefly, One-Step RT-ddPCR Mastermix (Bio-Rad) was mixed with manganese acetate solution (1 mM), primers/probe mix (0.9 µM primers and 0.25 µM probe; Integrated DNA Technologies), and RNA sample (approximately 60 ng/µl for HIV-1 mRNA and 0.60 ng/µl for *TBP* mRNA) in a total volume of 20 µl. This mix reaction was loaded into a Bio-Rad droplet generator cartridge along with 70 µl of droplet generation oil. The cartridge was placed in the droplet generator QX100™ device (Bio-Rad). Droplets were generated following the manufacturer's instructions and transferred to a 96-well PCR plate, which was subsequently sealed using PX1™ PCR plate sealer (Bio-Rad). RNA was retro-transcribed and amplified using the C1000 Touch™ Thermal Cycler (Bio-Rad) (retro-transcription: 60, 30'; enzyme activation: 95ºC, 5'; 40 cycles: 94ºC, 30" and 60ºC, 60"; final extension: 98ºC, 10'; 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 (Fig. 14).

PBMCs from healthy donors were used as negative controls to set the positive/negative threshold for ddPCR analysis. For each sample and control well, 2 replica wells were used. Moreover, each RNA sample was amplified using ddPCR Mastermix instead of One-Step RT-ddPCR Mastermix as a control of DNA contamination. None of these wells showed positive droplets, demonstrating no contamination with DNA.

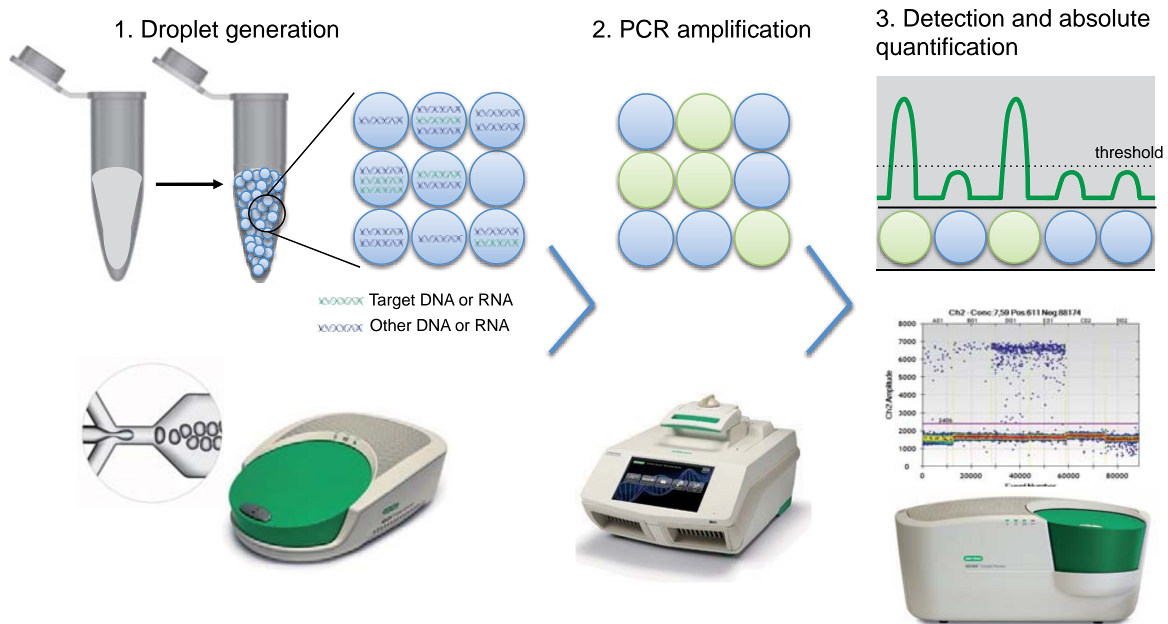


Figure 14. Schematic representation of droplet digital polymerase chain reaction (ddPCR) in order to quantify proviral HIV-1 DNA, 2-LTR circles or cell-associated HIV-1 mRNA

3A.2.5. Ultrasensitive plasma viremia quantification

To evaluate viral load (VL) below 50 copies/ml, up to 7.5 ml of plasma were ultracentrifuged at 170,000xg at 4ºC for 30 min. before viral RNA extraction using the m2000sp Abbott device. HIV-1 RNA copies were quantified in the Abbott m2000rt instrument using the Abbott Real-Time HIV-1 Assay (Abbott Molecular Inc.) and the laboratory-defined applications software from the instrument. HIV-1 RNA copies in the low range were determined by means of a calibration curve set between 1,000 and 10 copies/ml (five points at 1/3 dilutions: 1,000, 300, 100, 30, and 10 in triplicate). The quantification method was validated in triplicate with a positive control (prequantified standard HIV-1 from the World Health Organization) in the range of 128-0.5 copies/ml (9 serial 1/2 dilutions). The concentration protocol was validated by running direct *versus* diluted ultracentrifuged pre-quantified plasma samples, with VL ranging from 400 to 10 HIV-1 RNA copies/ml (Fig. 15).

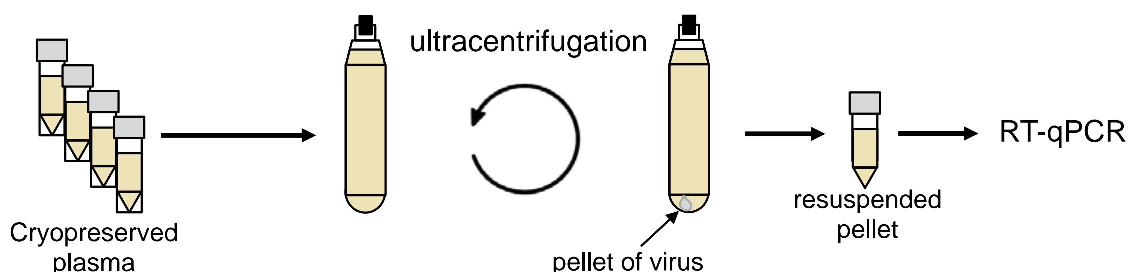


Figure 15. Schematic view of the procedure to quantify the ultrasensitive viral load from plasma samples of HIV-1 infected ART suppressed patients

3A.2.6. Quantitative viral outgrowth assay (qVOA) determination

To measure the replication-competent reservoir, we performed a limiting dilution cell culture assay as previously described (102), with minor modifications. Specifically, fresh $CD4^+$ T cells from HIV-1 infected patients [$(1.5\text{-}13)\times10^6$] were cultured in serial dilutions (Fig. 16). For each dilution we performed 2 replicas. Then, $CD4^+$ T cells were co-cultured with irradiated PBMCs in the presence of phytohemagglutinin (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 (known as 3×3 cells) were added to the co-culture (104,105). Cell culture medium (Rowell Park Memorial Institute (RPMI)-1640 (Gibco, Invitrogen) containing 1% Penicillin/Streptomycin (P/S; Gibco, Invitrogen), 10% FBS, 100 U/ml IL-2 and 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 12 were used to measure $p24^{Gag}$ with ELISA (Fig. 15). The frequency of infectious HIV-1 units per million $CD4^+$ T cells (IUPM) was determined using IUPMStats v.0.3 (Rosenbloom's software), based on the maximum likelihood method (184).

TCGF were produced using PBMCs isolated from buffy coats of HIV-1 seronegative donors purchased from the Banc de Sang i Teixits (BST; Barcelona, Spain). PBMCs were obtained by Ficoll-Hypaque density gradient (Fig. 12), resuspended at a concentration of 6×10^6 cells/ml in RPMI containing 1% P/S and 2.5% Human AB serum heat inactivated (Invitrogen, Life Technologies), and cultured in T150 flasks (Corning®). After 24 hours in culture, 2 µg/ml of PHA and 5 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) were added to each flask. Then, cells were incubated for 4 hours at 37ºC, washed twice with PBS containing 2 mM EDTA and 2% FBS, resuspended in 70 ml of the previous medium and cultured at 37ºC. After 40 hours, TCGF dissolved in culture supernatants were harvested, centrifuged at 400xg for 10 min., filtered through 0.45 nm filters (Millipore) and stored at -20ºC until use.

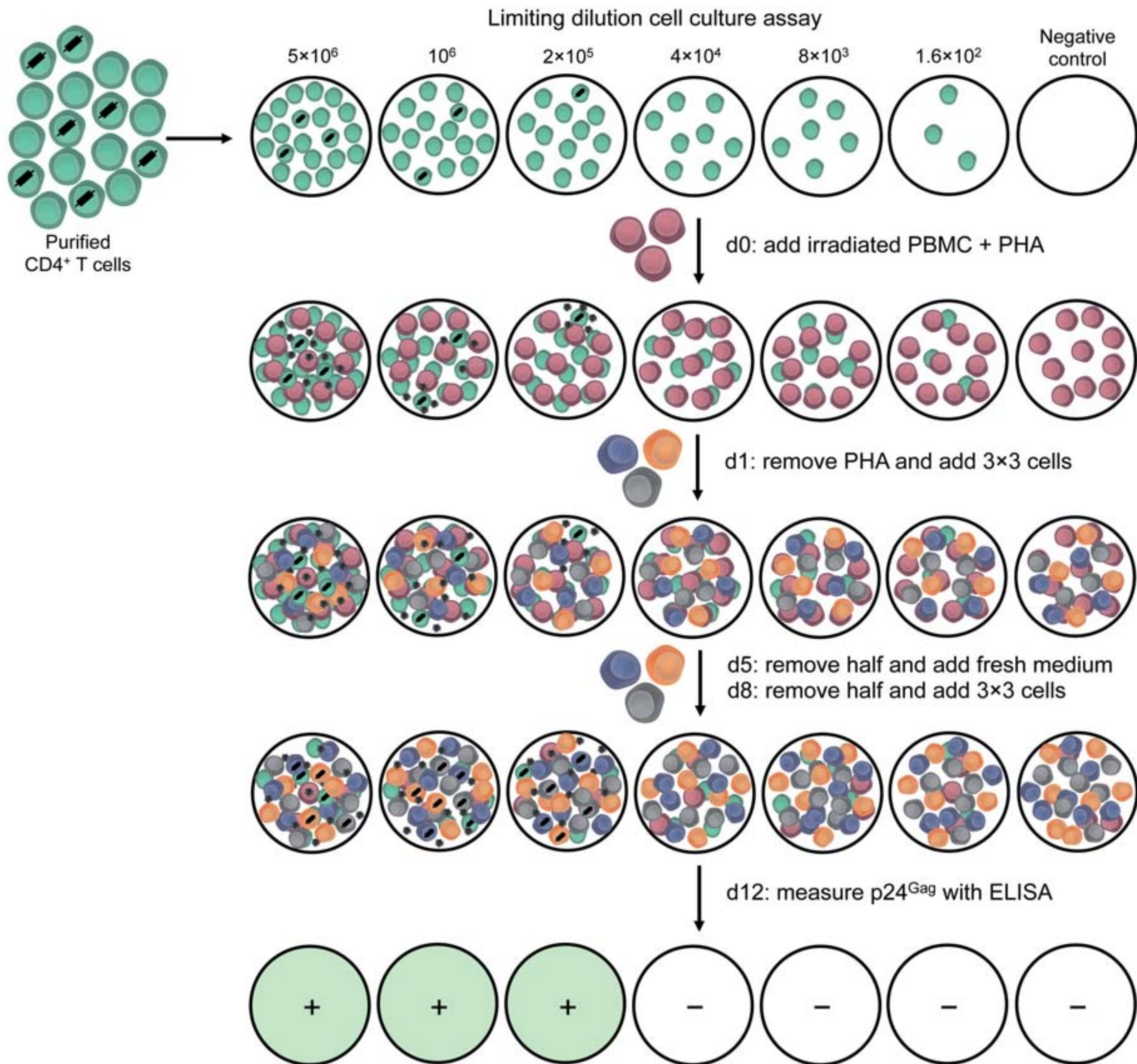


Figure 16. Schematic representation of quantitative viral outgrowth assay (qVOA)

Irradiated PBMCs were prepared using buffy coats from HIV-1 seronegative donors. Briefly, PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation (Fig. 12), inactivated by γ-irradiation (50 Gy in Cs-source irradiator) and resuspended at a concentration of $2.5×10^6$ cells/ml in RPMI containing 2% P/S, 10% FBS and 0.5 µg/ml PHA. Cells were stored in ice until co-culturing with HIV-1 infected $CD4^+$ T cells.

3×3 cells were prepared using buffy coats from 3 different HIV-1 seronegative donors. Specifically, the blood was depleted of $CD8^+$ T cells with RossetteSep Human CD8 Depletion Cocktail (StemCell) in order to avoid CD8 cross-reactivity between the different donor-cells. Then, CD8-depleted PBMCs from the 3 donors were isolated by Ficoll-Hypaque density gradient centrifugation (Fig. 12), collected

together in a falcon tube, counted with perfect-count microspheres™, resuspended in RPMI with 2% P/S and 10% FBS, split in 3 flasks, and cultured for 72 hours. One flask was previously seeded with OKT3 (antibody α-CD3) and the other flasks contained 5 or 0.5 µg/ml of PHA in the medium, in order to obtain CD8-depleted PBMCs in different activation conditions. After the 3 days of culture, the cells were mixed, counted with perfect-count microspheres™, resuspended at a concentration of 0.67×10^6 cells/ml in RPMI containing 1% P/S, 10% FBS, 100 U/ml IL-2 and 2% TCGF; and co-cultured with the HIV-1 infected $CD4^+$ T cells and the irradiated PBMCs. On day 8 of the assay 3×3 cells were added at a concentration of 10^6 cells/ml.

3A.2.7. Proviral HIV-1 DNA quantification

To evaluate the size of the proviral reservoir, peripheral $CD4^+$ T cells (5×10^6) were resuspended in lysis buffer at a concentration of 50,000 cells/µl. Lysis buffer 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 more than 10 hours at 55ºC, and proteinase K was inactivated at 95ºC for 5 min. Lysed $CD4^+$ T cells extracts were used to measure cell-associated total HIV-1 DNA by ddPCR with 5'LTR (73) and Gag (93) primers/probes sets (Table 1). The *RPP30* cellular gene was quantified in parallel to normalize sample input (185) (Table 1). Briefly, ddPCR supermix for probes (Bio-Rad) was mixed with primers/probe mix (0.9 µM primers and 0.25 µM probe; Integrated DNA Technologies), and 2 µl of cell lysate (equivalent to 100,000 cells for HIV-1 DNA and to 2,000 cells for *RPP30*) in a total volume of 20 µl. This mix reaction was loaded into a Bio-Rad droplet generator cartridge and the droplets were generated as previously described for cell-associated HIV-1 RNA. DNA was amplified using the C1000 Touch™ Thermal Cycler (Bio-Rad) (initial denaturation: 95ºC, 10'; 40 cycles: 94ºC, 30" and 57ºC, 60"; final extension: 98ºC, 10'; hold: 4ºC), and, after cycling, droplets were analyzed immediately 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) (Fig. 14).

For each sample, 2 to 3 replica wells were used. PBMCs from healthy donors were used as negative controls and assayed in each plate to set the posi-

tive/negative threshold for ddPCR analysis, as for cell-associated HIV-1 RNA. The number of those negative control wells was the same as the replicas for each sample.

3A.2.8. HIV-1 2-LTR circles quantification

To estimate the level of residual new infections, lysed $CD4^+$ T cells extracts were used to quantify the number of 2-long terminal repeat (2-LTR) circles by ddPCR with 2-LTR primers/probe set (78) (Table 1), following the same protocol as for proviral HIV-1 DNA (Fig. 14).

Table 1. Primer and probe sequences

Name	Sequence (5'-3')	Strand	Target	Ref.
LTR-U5 integrated	GTTCGGGCGCCACTGCTAG	Forward	Total DNA & CA-RNA	(73)
LTR-R integrated	TTAAGCCTCAATAAAGCTTGCC	Reverse		
New integrated-2 Probe	CCAGAGTCACACAACAGACGGGCA	Probe		
HIV_F (SCA)	CATGTTTTCAGCATTATCAGAAGGA	Forward		(93)
HIV_R (SCA)	TGCTTGATGTCCCCCCACT	Reverse		
HIV Probe (SCA)	CCACCCCACAAGATTTAAACACCATGCTAA	Probe		
New C1	CTAACTAGGGAACCCACTGCT	Forward	2-LTRs	(78)
C4R	GTAGTTCTGCCAATCAGGGAAG	Reverse		
2nr4nr Probe	AGCCTCAATAAAGCTTGCCTTGAGTGC	Probe		
RPP30-F	GATTTGGACCTGCGAGCG	Forward	*RPP30*	(185)
RPP30-R	GCGGCTGTCTCCACAAGT	Reverse		
RPP30 Probe	CTGACCTGAAGGCTCT	Probe		
TBP-S	TTCGGAGAGTTCTGGGATTGTA	Forward	*TBP*	(183)
TBP-AS	TGGACTGTTCTTCACTCTTGGC	Reverse		
TBP-Probe	CCGTGGTTCGTGGCTCTCTTATCCTCA	Probe		

3A.2.9. mRNA expression of APOBEC3G, TRIM5α, BST2, and TRIM22

RNA extracted from $CD4^+$ T cells (1×10^6) was transcribed into cDNA using TaqMan® Reverse Transcription Reagents (Applied Biosystems). cDNA was amplified using TaqMan® Gene Expression Assay primer/probe sets (Applied Biosystems) for the panel of genes and for the housekeeping gene *GUSB* (186). Thermal cycling was performed using the Comparative C_T ($\Delta\Delta C_T$) program of the 7500 RT-PCR System (Applied Biosystems), and the data were analyzed using 7500 RT-PCR Software (Applied Biosystems). Raw C_T numbers of amplified gene products were normalized to the passive reference ROX. ΔC_T data were obtained by normalizing the samples with expression of the housekeeping gene, *GUSB*. Fold induction ($\Delta\Delta C_T$) was determined by normalizing expression at d28 relative to d0.

3A.2.10. Distribution and activation of T-cell subsets.

T-cell immunophenotyping was performed in cryopreserved PBMCs. The cells were thawed and washed in PBS containing 10% FBS. PBMCs (2×10^6) were stained with Viability Dye eFluor 506 (eBioscience) for 30 min at 4ºC, washed and stained for 20 min at 4ºC with the following antibodies: CD3 (APC-Cy7), CD4 (PerCP), CD8 (AF700), CD45RA (FITC), CD27 (BV605), CCR7 (PE-Cy7), CD38 (PE-CF594), HLA-DR (APC), PD-1 (BV421) from Becton Dickinson and Siglec-1 (PE) from bioNova. Controls with combinations of CD3, CD4, and CD8 antibodies were established for all samples, and fluorescence minus one (FMO) controls were used to set gates for activation markers. Data were acquired in an LSR-Fortessa flow cytometer (Becton Dickinson) coupled to an HTS loader. At least 200,000 T cells were collected for each sample.

T-cell and monocyte gates were first defined according to morphological parameters and cell viability. Living $CD3^+$ T cells were classified into $CD4^+$ and $CD8^+$ T cells. Finally, T-cell subpopulations were classified by the expression of the markers CD45, CCR7, and CD27, as follows: T_N (naïve: $CD45^+CCR7^+CD27^+$ cells), T_{CM} (central memory: $CD45^-CCR7^+CD27^+$ cells), T_{TM} (transitional memory: $CD45^-CCR7^-CD27^+$ cells), T_{EM} (effector memory: $CD45^-CCR7^-CD27^-$ cells), and T_{EMRA} (terminally differentiated effector memory: $CD45^+CCR7^-CD27^-$ cells). Activation markers (CD38, HLA-DR) and exhaustion markers (PD-1) were analyzed within each T-cell subset. The percentage of Siglec-1^+ monocytes ($CD4^+CD3^-$ cells) was also analyzed. Analysis was carried out using FlowJo software 9.2.3v (Tree Star).

3A.2.11. Statistical analysis

Differences between groups were analyzed using the Mann-Whitney and Kruskal-Wallis tests. Changes within a group were evaluated using the Wilcoxon signed-rank test. For categorical variables, independence between groups was studied using the Fisher exact test. The Wald test was used to compare slopes between groups in a mixed regression model. The Spearman test and Winsor correlation test were used to evaluate the association between variables in independent and paired samples, respectively. Censored matched pairs of ultrasensitive viral load were analyzed using the paired Prentice-Wilcoxon test. Statistical signifi-

cance was set at 5% for all the tests. The analyses were performed with R (v3.0.2), GraphPad Prism (v5.01) and MiniTab® (v16.2.3).

3A.3. Results

3A.3.1. Patients

To evaluate the short-term effect of IFNα on levels of persistent HIV-1 during suppressive ART, we prospectively took blood samples from patients initiating IFNα-RBV as treatment for HCV infection.

The final study population comprised 21 HIV/HCV-coinfected patients recruited at Hospital Vall d'Hebron and Hospital Germans Trias i Pujol. A patient of the control group with detectable viremia at study d0 was excluded from the final analysis.

The demographic and clinical characteristics of patients included in the IFN group and the control group were comparable. Most patients were men, with a median age of 47 years, HIV-1 infection for more than 20 years, and HIV-1 suppression on ART maintained for a median of 4.5 years (Table 2).

No serious adverse events were reported, and the initial dose was maintained during the study.

3A.3.2. Cell-associated HIV-1 mRNA and residual viremia

The quantification of cell-associated HIV-1 RNA revealed low-level viral transcription in peripheral $CD4^+$ T cells in all patients. The median expression of HIV-1/*TBP* in $CD4^+$ T cells decreased 9-fold at d28 in IFNα-treated patients ($p=0.049$), whereas no significant changes were observed in the control group (Table 3, Fig. 17A).

We measured residual plasma viremia to evaluate whether the decline in cell-associated viral transcription decreased production of virus. We detected plasma viremia in 90% of patients. However, we observed no significant changes in any group during the study (Table 3, Fig. 17B), suggesting that short-term treatment with IFNα does not reduce residual viremia, despite its effect on viral transcription.

Table 2. Patient characteristics and clinical results

	Control group (n=10)	*IFN group (n=10)*	*p value between groups*
Age (years), median [IQR][a]	47 [45-53]	47 [41-51]	0.430
Women, n (%)[b]	1 (10)	2 (20)	1.000
2 drug–based regimen, n (%)[b]	2 (20)	4 (40)	0.629
3 drug–based regimen, n (%)[b]	8 (80)	6 (60)	
PI-based regimen, n (%)[b]	8 (80)	7 (70)	1.000
Time since diagnosis (years), median [IQR][a]	23.5 [20.2-24.0]	24.0 [19.8-25.2]	0.570
Duration of suppression of HIV-1 VL (years), median [IQR][a]	4.5 [2.5-5.8]	4.5 [2.2-7.8]	0.940
HCV VL			
IU/l d0, median [IQR][a]	4.1×10^6 [$(1.9\text{-}4.9) \times 10^6$]	2.5×10^6 [$(1.3\text{-}4.5) \times 10^6$]	0.190
IU/l d28, median [IQR][a]	NA	3.7×10^4 [$(0.2\text{-}77.8) \times 10^4$]	NA
p value (d0 vs. d28)[c]	NA	**0.049**	
$CD4^+$ T-cell count			
Absolute (cells/µl) d0, median [IQR][a]	447 [295-670]	437 [318-624]	0.820
Absolute (cells/µl) d28, median [IQR][a]	570 [480-650]	310 [203-370]	**0.010**
p value (d0 vs. d28)[c]	1.000	**0.009**	
Percentage d0, median [IQR][a]	21.5 [18.3-30.8]	22.5 [21.0-37.5]	0.450
Percentage d28, median [IQR][a]	25.0 [22.0-33.0]	28.1 [22.5-36.7]	0.760
p value (d0 vs. d28)[c]	1.000	0.444	
$CD8^+$ T-cell count			
Absolute (cells/µl) d0, median [IQR][a]	1,050 [1,010-1,200]	650 [585-1,130]	0.370
Absolute (cells/µl) d28, median [IQR][a]	1,190 [660-1,310]	440 [378-523]	**0.005**
p value (d0 vs. d28)[c]	0.813	**0.031**	
Percentage d0, median [IQR][a]	54.6 [42.2-60.0]	49.0 [38.7-58.9]	0.620
Percentage d28, median [IQR][a]	56.0 [37.0-59.0]	43.5 [39.2-57.9]	0.850
p value (d0 vs. d28)[c]	0.892	0.652	

[a] p value between groups*: Kruskal-Wallis test* (GraphPad Prism v5.01).
[b] p value between groups: *Fisher exact test* (R v3.02).
[c] p value (d0 vs. d28): *Wilcoxon signed-rank test* (GraphPad Prism v5.01).
IQR. interquartile range; PI. protease inhibitor; VL. viral load; IU. infectious units

3A.3.3. Replication-competent HIV-1 reservoir, total HIV-1 DNA, and 2-LTR DNA circles

To evaluate the effect of IFNα on the size of the replication-competent reservoir, we measured the infectious units per million cells (IUPM) in a qVOA of $CD4^+$ T cells. We detected replication-competent viruses in 90% of patients. However, we did not detect a significant decrease at d28 in any group (Table 3, Fig. 17C).

We also detected circulating $CD4^+$ T cells harboring proviral DNA in all patients. The count remained stable in both study groups (Fig. 17D).

We quantified the number of 2-LTR circles to estimate the level of residual replication. We detected HIV-1 episomes in 90% of patients. This parameter remained stable in both study groups (Fig. 17E).

Taken together, the data suggest that short-term treatment with IFNα did not affect the replication-competent HIV-1 reservoir, the total proviral HIV-1 reservoir, or the number of residual new infections.

3A.3.4. Expression of IFNα-stimulated genes (ISGs)

As most of the genes up-regulated by IFNα are host restriction factors, we analyzed the effect of IFNα on the restriction factors TRIM22, BST2, TRIM5α, and APOBEC3G, all of which inhibit HIV-1 replication. The mRNA expression of all these host restriction factors increased at d28 of treatment with IFNα-RBV (Table 3, Fig. 18B). Conversely, no changes were detected in the control group. Moreover, we observed significant differences between the groups in the expression of TRIM22 and TRIM5α at d28 ($p<0.001$ and $p=0.025$, respectively; Table 3, Fig. 18B).

Given that Siglec-1 is a type I IFNα–induced protein that plays a key role in HIV-1 spread through mDC-CD4 synapsis (187), we measured changes in expression of Siglec-1 in monocytes. We observed a significant increase in Siglec-1 protein expression at d28 ($p=0.002$), with significant inter-group differences ($p<0.001$) (Table 3, Fig. 18A).

Table 3. Effect of IFNα-RBV on markers of HIV-1 and IFNα-stimulated gene expression

	Control group (n=10)	*IFN group (n=10)*	*p value between groups*
Cell-associated HIV-1 RNA			
Ratio HIV-1 RNA/housekeeping d0, median [IQR][a]	0.13 [0.07-1.45]	0.92 [0.23-3.29]	0.143
Ratio HIV-1 RNA/housekeeping d28, median [IQR][a]	0.13 [0.05-0.42]	0.25 [0.02-0.96]	0.912
p value (d0 vs. d28)[b]	0.432	**0.049**	
Ultrasensitive pVL			
Copies/ml plasma d0, median [IQR][a]	0.90 [0.20-6.33]	2.40 [0.88-4.65]	0.463
Copies/ml plasma d28, median [IQR][a]	0.20 [0.20-3.53]	0.65 [0.20-1.65]	0.967
p value (d0 vs. d28)[c]	0.147	0.108	
Infectious units per million (IUPM) $CD4^+$ T cells			
IUPM d0, median [IQR][a]	0.42 [0.23-8.29]	1.61 [0.23-4.45]	0.676
IUPM d28, median [IQR][a]	0.42 [0.10-12.59]	1.11 [0.40-2.02]	0.870
p value (d0 vs. d28)[b]	0.813	0.359	
Total HIV-1 DNA			
Copies/10^6 $CD4^+$ T cells d0, median [IQR][a]	1,302 [606-2,879]	1,425 [561-2,567]	0.853
Copies/10^6 $CD4^+$ T cells d28, median [IQR][a]	1,274 [594-1,860]	1,669 [589-2,985]	0.393
p value (d0 vs. d28)[b]	0.065	0.131	
2LTR HIV-1 DNA			
Copies/10^6 $CD4^+$ T cells d0, median [IQR][a]	10.7 [0-15.7]	4.5 [0-15.7]	0.847
Copies/10^6 $CD4^+$ T cells d28, median [IQR][a]	4.8 [2.3-10.8]	8.5 [2.7-34.7]	0.383
p value (d0 vs. d28)[b]	0.426	0.138	
IFN*α*-stimulated genes fold change			
TRIM22 d28, median [IQR][d]	1.15 [0.81-1.38]	2.02 [1.58-3.07]	**<0.001**
p value (d0 vs. d28)[b]	0.322	**0.002**	
BST2 d28, median [IQR][d]	1.04 [0.81-1.20]	1.96 [1.17-2.43]	0.0674
p value (d0 vs. d28)[b]	0.922	**0.037**	
TRIM5α d28, median [IQR][d]	0.96 [0.66-1.19]	1.67 [1.27-1.98]	**0.025**
p value (d0 vs. d28)[b]	0.695	**0.002**	
APOBEC3G d28, median [IQR][d]	0.89 [0.68-1.17]	1.24 [1.13-1.52]	0.0912
p value (d0 vs. d28)[b]	0.432	**0.004**	
Siglec-1 percentage[e] d28, median [IQR][d]	1.01 [0.89-1.34]	1.54 [1.13-3.63]	**0.016**
p value (d0 vs. d28)[b]	0.492	**0.002**	
Siglec-1 GeoMean d28, median [IQR][d]	1.09 [0.86-1.33]	5.85 [3.69-15.02]	**<0.001**
p value (d0 vs. d28)[b]	0.432	**0.002**	

[a] p value between groups*: Kruskal-Wallis test* (GraphPad Prism v5.01).
[b] p value (d0 vs. d28): *Wilcoxon signed-rank test* (GraphPad Prism v5.01).
[c] p value (d0 vs. d28): *Paired Prentice-Wilcoxon test* (MiniTab® v16.2.3).
[d] p value between groups: *Wald test* (R v3.02).
[e] percentage of cells expressing Siglec-1 protein

Figure 17. Impact of IFNα/RBV on viral parameters in CD4$^+$ T cells. The IFN group is shown in dark cyan and the control group in light blue. Individual patient dynamics are represented over the boxplot. Solid black dots represent Tukey outliers. **(A)** HIV-1 mRNA transcription, **(B)** residual plasma viremia, **(C)** IUPMs, **(D)** total HIV-1 DNA, **(E)** 2-LTR DNA circles. The dotted line represents the limit of detection (LOD=0.2 HIV-1 RNA copies/mL; LOD= –1.260 $\log_{10}$ (IUPM); LOD=3 2-LTR copies/10^6 CD4$^+$ T cells). Solid grey dots represent data below the LOD. Significant and nonsignificant (n.s.) p values for longitudinal comparisons (Wilcoxon signed-rank test [GraphPad Prism v5.01] and the paired Prentice-Wilcoxon test [Minitab® v16.2.3]) are shown.

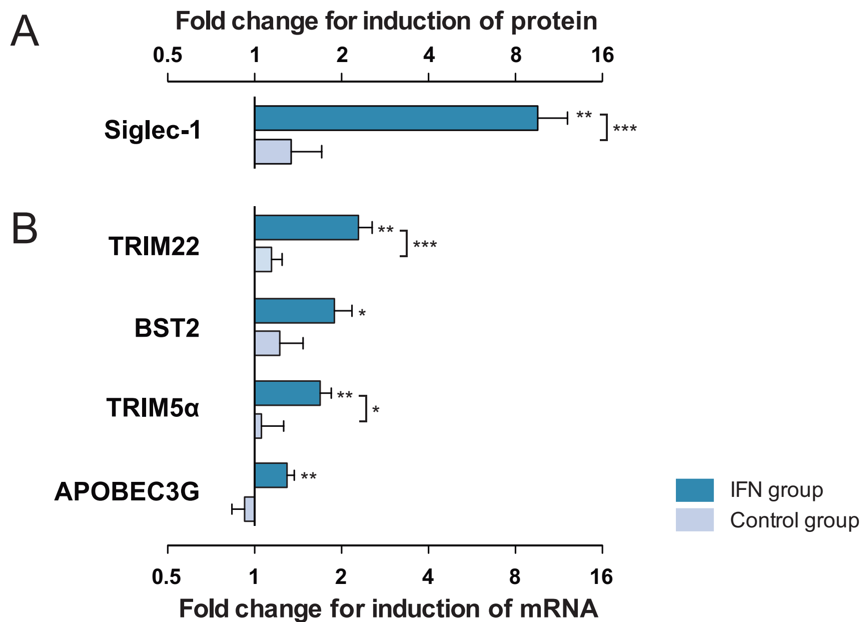


Figure 18. Impact of IFNα-RBV on IFN-stimulated genes. **(A)** Fold change for induction of Siglec-1 in monocytes and **(B)** mRNA fold change for induction of the host restriction factors TRIM22, BST2, TRIM5α, and APOBEC3G in $CD4^+$ T cells are represented at d28 after administration of IFNα-RBV. The graph shows mean and SEM values. Asterisks denote significant differences from baseline (Wilcoxon signed-rank test, GraphPad Prism v5.01) or significant differences between group slopes in a mixed regression model (Wald test, R v3.02: *$p<0.05$, **$p<0.01$, ***$p<0.001$).

3A.3.5. Analysis of $CD4^+$ T-cell subsets, activation and exhaustion markers

Significant changes in absolute $CD4^+$ and $CD8^+$ T-cell counts were seen in the IFN group between d0 and d28 (p=0.009 and p=0.031, respectively) and between the groups at d28 (p=0.010 and p=0.005; Table 2). In $CD4^+$ T cells, a significant decrease was observed in all subsets except for T_{EMRA} cells. The analysis of the subset frequencies showed a slight increase in the T_{CM} and T_{EM} subsets (p=0.048 and p=0.037, respectively) and a reduction in the T_{TM} subpopulation (p=0.037) in the IFNα group at d28 of treatment. However, none of the differences were statistically significant between the groups.

A general decrease in the expression of activation markers was observed in $CD4^+$ T cells from IFNα-treated patients (Fig. 19A). The frequency of HLA-DR^+CD38^+ was significantly lower in the IFN group than in the control group for the whole $CD4^+$ T-cell population and for all the subsets except T_{EMRA} cells (Fig. 19A). Conversely, the frequency of HLA-DR^+CD38^+ cells remained unchanged in the whole $CD8^+$ T-cell population (data not shown). We did not observe any significant difference in PD-1 percentages in the treated group, either for total $CD4^+$ T cells or

for individual subsets (Fig. 19B). The data suggest that short-term treatment with IFNα reduces peripheral CD4+ T-cell activation.

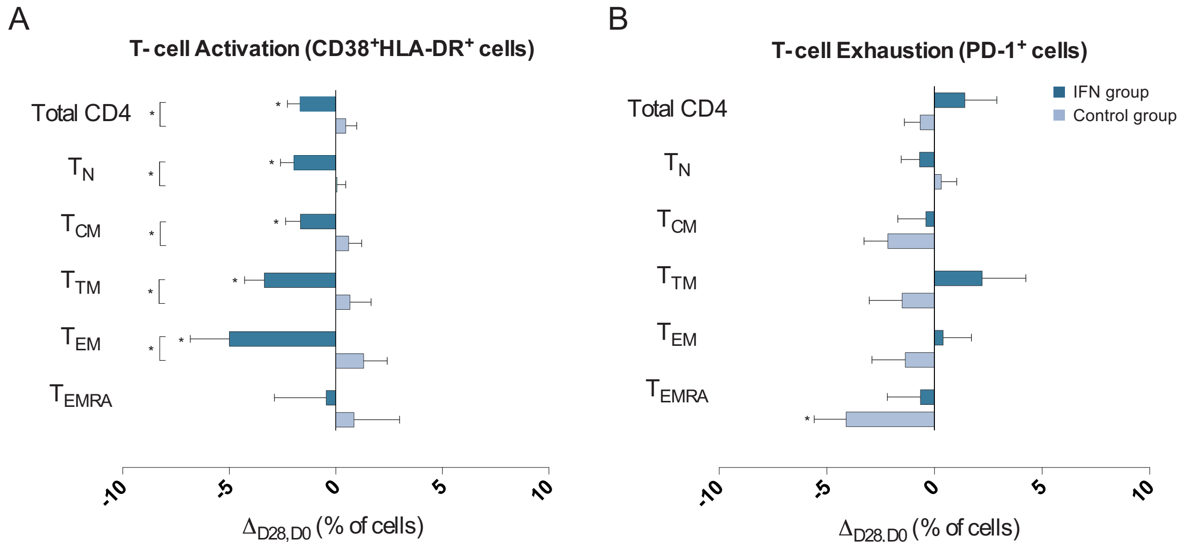


Figure 19. Impact of IFNα-RBV on CD4+ T-cell activation and exhaustion markers. Changes in the percentage of HLA-DR+CD38+ cells **(A)** and PD-1+ cells **(B)** in the whole CD4+ T-cell compartment and within each defined subset. Asterisks denote significant differences from baseline (Wilcoxon signed-rank test, GraphPad Prism v5.01) or significant differences between groups (Wald test, R v3.02: *p<0.05, **p<0.01, ***p<0.001).

3A.4. Discussion

Our study is the first to evaluate the short-term effect of IFNα-RBV on multiple virological and immunological parameters generally evaluated during interventions to reduce the HIV-1 reservoir.

Quantification of cell-associated HIV-1 RNA at baseline revealed persistent low-level viral transcription in peripheral $CD4^+$ T cells in all patients, despite receiving suppressive ART for a median of 4.5 years. Moreover, treatment with IFNα-RBV decreased transcription of HIV-1 RNA in circulating $CD4^+$ T cells while simultaneously reducing T-cell activation and increasing expression of IFN-stimulated genes (ISGs). It seems that the decrease in T-cell activation could contribute to a decrease in viral transcription and mRNA translation (188). However, these changes could also be associated with IFNα-driven migration of T cells from peripheral blood to lymphoid tissue (189). Although we observed slight changes in the relative frequency of $CD4^+$ T-cell subsets, it remains to be determined which T-cell subsets mainly contribute to HIV-1 RNA transcription under ART.

Up-regulation of host restriction factors induced by IFN is expected to reduce viral replication. In our study, TRIM22, a nuclear restriction factor able to inhibit HIV-1 transcription (190), was markedly up-regulated in IFNα-treated patients, potentially contributing to the decrease in viral transcription.

Notwithstanding, the lack of changes in ultrasensitive plasma viremia suggests that the effect of short-term IFNα on peripheral $CD4^+$ T cells with decreased viral transcription may not extend to all tissue cells that contribute to residual plasma viremia.

In contrast with the results from previous studies where IFNα was administered for longer than 28 days (180,181), we did not detect any change in the size of the HIV-1 reservoir. Treatment with IFNα-RBV was previously shown to lead to a moderate but significant and sustained decline in HIV-1 DNA in $CD4^+$ T cells from HIV/HCV–coinfected patients receiving ART (181). Moreover, Azzoni *et al.* (180) found a drop in integrated HIV-1 DNA in IFNα-treated subjects who controlled viral rebound after interruption of ART. Furthermore, although a significant decrease in 2-LTR circles was reported in patients on suppressive ART treated with IFNα-RBV

(182), levels of episomes remained unchanged in the present study. Thus, the short period of treatment in our study (28 days) might explain these differences in total proviral DNA and 2-LTR levels, suggesting that administration of IFNα-RBV could have a differential effect over time. Moreover, we observed no correlation between the virological variables analyzed. We cannot exclude, however, that some of the negative findings in this study could also be due to issues regarding sample size and assay precision.

We conclude that 28 days of treatment with IFNα can trigger a reduction in HIV-1 transcription in HIV/HCV–coinfected patients on suppressive ART through up-regulation of viral restriction genes, mainly *TRIM22*, and a decrease in T-cell activation (Fig. 20). Although neither residual viremia nor replication-competent virus levels changed at d28 of treatment, the effects of longer treatment periods remain to be determined. Therefore, after 28 days of treatment, IFNα acts more as a viral suppressor than as a reactivating agent. Studies on long-term therapy with IFNα in patients on ART are necessary to elucidate the mechanism by which IFNα-mediated viral suppression extends from cell-associated RNA to DNA and whether it affects replication-competent reservoir levels.

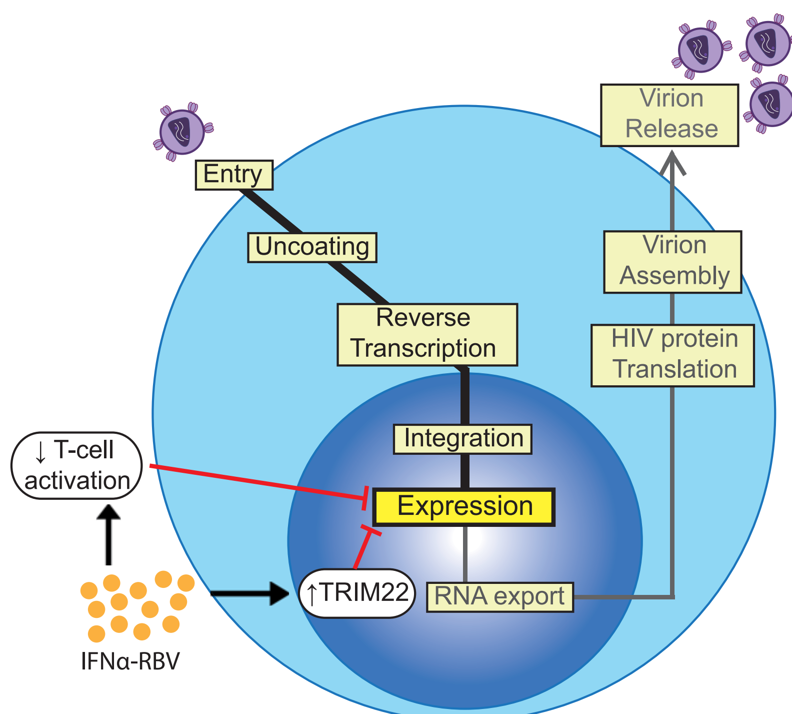


Figure 20. Schematic representation of interferon-α combined with ribavirine (IFNα-RBV) short-term effect in HIV-1 infected CD4^{+} T cell

CHAPTER 3B. Latency reactivation

This chapter corresponds to the manuscript:

Effect of lithium on HIV-1 expression and proviral reservoir size in the CD4^{+} T cells of antiretroviral therapy suppressed patients

Maria C. Puertas*, Maria Salgado*, Sara Morón-López, Dan Ouchi, Jose A. Muñoz-Moreno, José Moltó, Bonaventura Clotet and Javier Martinez-Picado

* These authors contributed equally to this work
S.M-L. second author

AIDS, 2014

3B.0. Presentation

This chapter evaluates the treatment with lithium carbonate as a latency reactivation approach to cure HIV-1 infection, due to its potential implication on HIV-1 provirus reactivation and, thus, its effect on the HIV-1 reservoir.

3B.1. Introduction

A reservoir of latently infected cells is involved in persistence of HIV-1 during suppressive antiretroviral therapy and is responsible for viral rebound after discontinuation of treatment. This viral reservoir is established early in acute infection and is maintained mostly in long-lived memory CD4^{+} T cells. Many therapeutic strategies have been proposed to reduce the size of this latent reservoir and thus achieve a functional cure; some of these strategies are based on disruption of viral latency, with the goal of inducing cell death or immune-mediated clearance (171).

Lithium is an inhibitor of GSK-3 (glycogen synthase kinase 3), which induces the intracellular accumulation of β-catenin and the subsequent activation of TCF/LEF-1 transcription factors (explained in Figure 21). This signaling pathway had been suggested to have an inhibitory effect on viral transcription in peripheral blood mononuclear cells (PBMCs) *in vitro* (191,192). However, recent data have shown the potential effect of β-catenin signaling on viral reactivation in latently infected cell lines and primary memory CD4^{+} T-cell cultures (193). Thus, the potential effect of this pathway on breaking HIV-1 latency *in vivo* is controversial. We took advantage of an ongoing pilot trial on lithium carbonate for treatment of HIV-1-associated neurocognitive abnormalities to investigate the effect of this drug on viral reservoir in ART-suppressed patients.

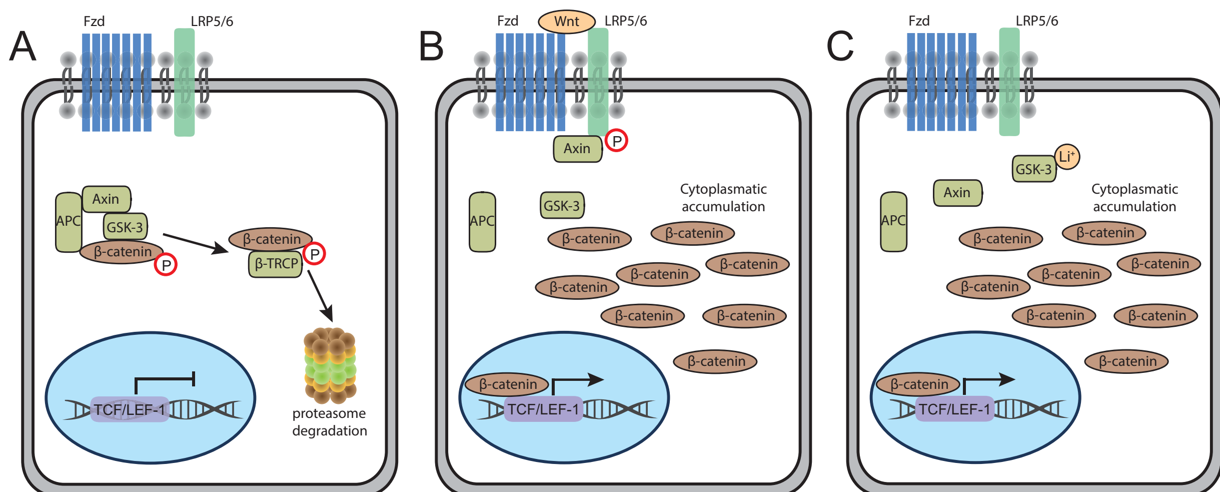


Figure 21. Schematic representation of Wnt/β-catenin signaling pathway. **(A)** In the absence of Wnt signals, the cellular concentration of free β-catenin is low, because a complex of the adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β) and axin protein is responsible for regulating the level of β-catenin, via GSK-3-mediated phosphorylation of specific serin and threonine residues in β-catenin and proteasome degradation. **(B)** Upon Wnt signaling, the activity of GSK-3β is inhibited, hence β-catenin is accumulated in the cytoplasm. The accumulated β-catenin can enter the nucleus and activates the target genes such as *TCF/LEF-1*. **(C)** Alternative pathway to Wnt signaling, using lithium carbonate ($(Li^{+})_2(CO_3)^{2-}$).

3B.2. Material and Methods

3B.2.1. Patients

This prospective proof-of-concept study included 9 HIV-1-infected patients taking suppressive ART (<50 HIV-1 RNA copies/mL). The patients started a 12-week course of lithium as part of the TRIANT-TE clinical study (NCT01348282), which aimed to compare the safety and efficacy of 2 pharmacological strategies in mild neurocognitive disorders, carried out at Hospital Germans Trias i Pujol (Badalona, Spain). The Institutional Review Committee approved the protocol and all participants provided written informed consent. Blood samples were collected at baseline and at weeks 2, 4, and 12.

3B.2.2. Cell-associated HIV-1 mRNA

To evaluate viral transcription levels, 2.5×10^6 $CD4^+$ T cells were purified from PBMCs and cell-associated HIV-1 mRNA was quantified, as previously described in chapter 3A.

3B.2.3. Proviral HIV-1 DNA

To evaluate the size of the proviral reservoir, lysed extracts from 1.5×10^6 $CD4^+$ T cells were used to measure cell-associated total HIV-1 DNA, as previously described in chapter 3A.

3B.2.4. Ultrasensitive plasma viremia

To evaluate viral load <50 copies/ml, up to 7.5 ml of plasma samples were ultracentrifuged at 50,000xg at 4ºC for 1 hour before quantification of HIV-1 RNA using the Cobas Ampliprep/Cobas Taqman HIV-1 test, version 2.0 (Roche). Serial dilutions of the positive control down to 5 HIV-1 RNA copies/ml were used to set up a new standard curve to infer quantitative values from raw RT-qPCR C_T data.

3B.2.5. Quantitative viral outgrowth assay (qVOA)

To quantify the size of the replication-competent reservoir, we performed a limiting dilution cell culture assay with $CD4^+$ T cells from HIV-1 infected subjects, as previously described in chapter 1.

3B.2.6. Statistical analysis

Longitudinal comparisons for cell-associated HIV-1 RNA and DNA were performed using GraphPad Prism (v5.01). Plasma viremia was analyzed using the paired Prentice-Wilcoxon test (MiniTab® v16.2.3).

3B.3. Results

3B.3.1. Patients

To evaluate the potential effect of lithium on the modulation of viral latency during suppressive ART, we prospectively took blood samples from patients initiating lithium carbonate as an experimental treatment for HIV-1-associated mild neurocognitive disorders.

Mean time from HIV-1 diagnosis was 10.7±6.5 years, and mean time of sustained virological suppression was 5.3±3.4 years. The initial lithium carbonate dose of 400 mg/day was subsequently titrated to reach blood levels of 0.4-0.8 mM, which were monitored throughout the study to assess treatment adherence. No serious adverse events were reported.

3B.3.2. Cell-associated HIV-1 mRNA and residual viremia

ddPCR revealed low-level viral transcription in circulating $CD4^+$ T cells in most patients (78%), despite years of undetectable viremia before study entry. Contrary to what was expected, two weeks after initiation of lithium therapy, cell-associated HIV-1 RNA transcripts decreased in 5 out of 6 patients (Fig 22A). A 40% reduction in viral transcription levels was observed at week 4. Of note, viral transcription increased later (p-value=0.03), and all patients had recovered their initial transcription pattern after 12 weeks of treatment.

We also evaluated whether changes at the intracellular level might have a perceptible impact on virus production. In order to detect slight changes in low-level plasma viremia, we quantified ultrasensitive viral load (detection limit of 1.3 HIV-1 RNA copies/mL). Most patients showed detectable viremia at study entry with this method (67%), although this proportion decreased to 44% immediately after initiation of lithium (Fig 22B), before rising again to 87% at week 12. Despite the fact that these data are not statistically significant, they paralleled the dynamics of HIV-1 expression in circulating $CD4^+$ T cells.

3B.3.3. Total HIV-1 DNA and replication-competent HIV-1 reservoir

To further evaluate the effect of lithium on the size and dynamics of the latent viral reservoir, we measured the proportion of circulating $CD4^+$ T cells harboring

proviral DNA. Initially, we detected a median of 1,173 HIV-1 copies per million CD4+ T cells (interquartile range: 388-2,343). However, this value decreased to 582 copies (373-1,606) after 4 weeks of treatment (Fig 22C). Longitudinal analysis revealed a statistically significant drop in the size of the proviral reservoir in CD4+ T cells at this point (19% median decrease; p-value=0.03). Nevertheless, this effect was not permanent, as longitudinal changes in total HIV-1 DNA were lost at week 12.

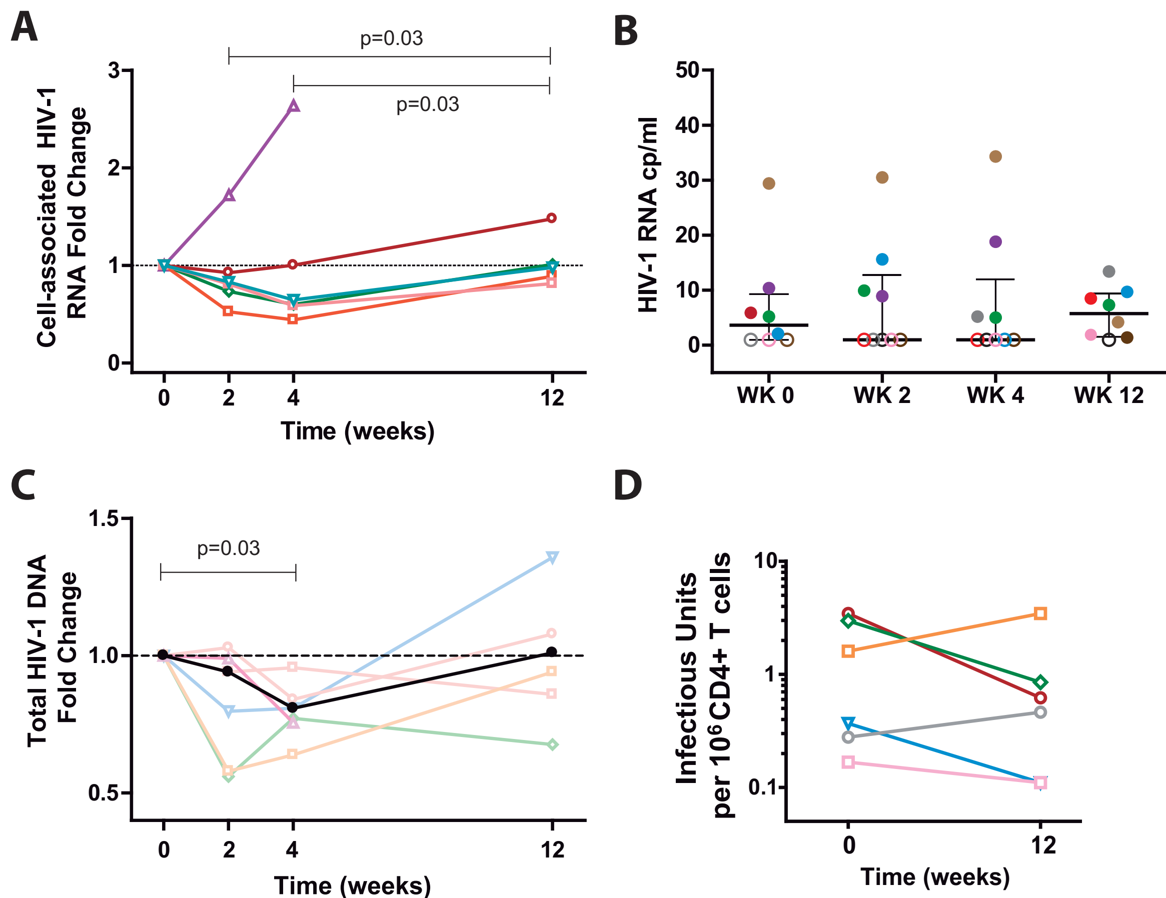


Figure 22. Effect of lithium on CD4+ T-cell viral production and HIV-1 reservoir size. **(A)** Dynamics of HIV-1 RNA transcription in circulating CD4+ T cells after administration of lithium. Fold changes with respect to baseline transcription levels are color-coded for each patient. **(B)** Plasma viremia as detected by ultrasensitive methodologies. Data from each patient are color-coded; open symbols represent undetectable samples (detection limit=1.3 HIV-1 RNA copies/mL). Median levels and interquartile ranges at each time-point are represented in black. **(C)** Total HIV-1 DNA reservoir size changes over time. Fold changes with respect to baseline are color-coded for each patient; the median fold change at each time-point of the study is represented in black. **(D)** Replicative-competent HIV-1 reservoir size, as measured in a viral outgrowth assay using CD4+ T cells, is color-coded for each patient at baseline and at the end of the study. Significant p-values for significant longitudinal comparisons are shown (Wilcoxon signed-rank test).

Although reversible effects had been observed in molecular measurements of total proviral reservoir size, potential long-term effects of lithium treatment on the replication-competent reservoir could not be ruled out. We addressed this question by measuring the frequency of infectious units per million CD4+ T cells (IUPM) in a

co-culture quantitative viral outgrowth assay (qVOA). Only samples from baseline and week 12 were analyzed. Although most patients tended to have decreased IUPM levels at week 12 (4 out of 6; Fig 22D), these changes did not reach statistical significance, neither at individual levels nor globally.

3B.4. Discussion

Lithium salts have been used extensively as mood stabilizers in the treatment of bipolar disorder. Lithium has also been used in HIV-1-infected patients to improve the course of secondary neurological disorders associated with neuroinflammation and viral persistence in the central nervous system. Nevertheless, little is known about the direct effect of lithium (including potential β-catenin signaling activation) on the different stages of viral replication in target $CD4^+$ T cells *in vivo*. Previous data showed an association between lithium treatment and reduced viral load in some HIV-1-infected patients (191). However, these retrospective analyses explored the use of lithium in the context of bipolar disorder, thus making it difficult to determine whether the reduction in viral load was solely attributed to the direct effect of lithium on infected cells or a concomitant effect of improved adherence to antiretroviral treatment following stabilization of the psychiatric disorder.

Lithium has also been recently reported to be useful for the treatment of HIV-1-associated neurocognitive impairment, which includes learning difficulties and reduced attention and memory capabilities (194,195). Thus, we based the present investigation on the design of an ongoing study evaluating lithium administered to HIV-1-infected patients who had been on effective ART for at least 1 year and had no major psychological disorders. The present proof-of-concept study is the first prospective analysis of the effect of lithium on HIV-1 expression and reservoir size in the $CD4^+$ T cells of virologically suppressed patients.

Lithium affected low-level viral transcription in circulating $CD4^+$ T cells but, contrary to our hypothesis, a significant decrease in cell-associated viral mRNA was observed in most patients at weeks 2 and 4 after initiation. This decrease was homogeneously observed in 5 out of 6 patients, confirmed by the HIV-1 RNA:DNA ratio (data not shown), and reflected in residual plasma viremia. This repressor effect of lithium is likely mediated by the β-catenin signaling pathway, including transcription factors from the TCF/LEF-1 family, which might bind to the HIV-1-LTR. Previous studies had identified TCF-4 as the downstream effector of the β-catenin signaling pathway involved in the inhibition of *in vitro* HIV-1 replication in PBMCs (191). Yet TCF-1 had been suggested to be able to mediate reactivation of latent HIV-1 transcription in several cell models, including primary memory

CD4$^+$ T cells (193). Indeed, TCF/LEF-1 transcription factors are differentially expressed depending on the cell type and maturation/activation status, as previously described for naïve versus memory T cell phenotypes (196). Our results indicate that, upon β-catenin signaling activation induced by lithium treatment, TCF/LEF-1 transcription factors with repressor activity might mediate the inhibition of residual viral production in CD4$^+$ T cells from virologically suppressed patients. In the case of memory CD4$^+$ T cells, β-catenin pathway has been described to be mechanistically related to self-renewal and survival, while activation seems to be suppressed (197). As CD4$^+$ T cells with central memory phenotype harbor most of HIV-1 DNA and mRNA detected in blood of ART-suppressed patients (61,198), further studies might assess the potential involvement of TCF-4 in the establishment of HIV-1-latency in these cells.

Unexpectedly, this lithium-mediated suppression of viral expression also led to a significant and fast decrease in proviral reservoir content in the CD4$^+$ T-cell population, suggesting a potential role for low-level viral production in supporting *de novo* infections and continuous replenishment of the viral reservoir during ART. Thus, lithium could alternatively be considered as a potential therapeutic agent aimed at inhibiting transcription from latently infected CD4$^+$ T cells. Actually, residual HIV-1 expression in persistent cellular reservoirs, regularly found in ART-suppressed patients, might also have a key role in maintaining the damaging immune activation driven by low-level viremia, thus making it a putative target for new therapeutic approaches aiming at achieving a functional cure.

The repressor effect of lithium was transient, and viral transcription and proviral levels were restored after 12 weeks of continuous treatment, possibly as a result of a cellular adaptive response to the chronic activation of the β-catenin signaling pathway. As in the case of other agents that also transiently modulate gene expression, future studies on improved administration strategies (e.g., intermittent doses or combination therapies) (199,200) are necessary to evaluate whether the unresponsiveness of target cells can be overcome, thus enhancing the potential of lithium to reduce the viral reservoir in the long term.

CHAPTER 3C. Therapeutic vaccination

This chapter corresponds to the manuscript:

Evaluation of the immunogenicity and impact on the latent HIV-1 reservoir of a conserved region vaccine, MVA.HIVconsv, in antiretroviral therapy-treated subjects

Gemma Hancock*, Sara Morón-López*, Maria C. Puertas, Eleni Giannoulatou, Annie Rose, Maria Salgado, Emma-Jo Hayton, Catharine Morgan, Brian Angus, Fabian Chen, Hongbing Yang, Javier Martinez-Picado, Tomas Hanke, Lucy Dorrell

* These authors contributed equally to this work
S.M-L. co-first author

Submitted

3C.0. Presentation

This chapter evaluates the administration of the HIVconsv immunogen vectored with the modified vaccinia Ankara virus (MVA) as a therapeutic vaccination approach to cure HIV-1 infection. This vaccine is based on conserved epitopes of different HIV-1 clades, which may elicit HIV-1 specific immune responses and impact the HIV-1 reservoir size in HIV-1 ART-suppressed patients.

3C.1. Introduction

Therapeutic immunization has attracted renewed interest as a strategy to enhance immune-mediated clearance of persistent HIV-infected $CD4^+$ T cells during long-term antiretroviral therapy (ART), with the goal of purging the latent viral reservoir to levels that are low enough to permit safe interruption of ART (201). Recently, vaccine candidates have been tested either as the sole adjunct to ART or in combination with ART and latency-reversing agents (LRAs) as a means to 'shock and kill' (202). In the first scenario, it is envisaged that vaccination would induce the expansion of HIV-specific effector T cells with the capacity to kill virus-producing $CD4^+$ T cells, thus reducing the frequency of latently infected cells and potentially delaying viral rebound during ART interruption (203,204). Immunization with a human adenovirus vector expressing HIV-1 Gag, a personalized vaccine based on autologous virus-pulsed dendritic cells or a Gag p24-based peptide vaccine, have shown modest yet statistically significant improvements in short-term virological control without ART (205–207). In the second scenario, it is hypothesized that pharmacological reactivation of $CD4^+$ T cells is needed to induce viral protein production and thus target the cell for immune recognition. This premise is supported by *in vitro* models of HIV-1 latency reversal; however, a poxvirus-vectored HIV-1 vaccine had only a transient effect on viral rebound whether tested alone or in combination with a candidate LRA, disulfiram, in a phase I clinical trial (139,208).

These studies have been pivotal in demonstrating the safety and feasibility of testing new vaccines and LRAs and in assessing short-term effects on viral reservoir parameters in ART-treated subjects. However, more potent agents will be needed in order to achieve therapeutic benefit. Most HIV-1 immunogens tested to date have been based on full-length HIV-1 proteins, an approach that may lead to preferential expansion of T cell clones targeting immunodominant and typically variable viral epitopes that have already escaped, or recapitulation of ineffective responses induced by natural infection (203,209). In the HIV-CORE 001 trial, we tested a conserved region immunogen, HIVconsv, vectored by modified vaccinia virus Ankara (MVA) in chronically infected ART-treated individuals. HIVconsv comprises 14 highly conserved regions of the viral proteome, assembled end-to-end as a chimeric protein and balanced for representation of the major clades, A-D

(Fig. 23)(210). By presenting the immune system with conserved HIV-1 sequences without the natural context of a full-length protein, we aimed to re-direct T cells towards viral epitopes that are vulnerable and typically sub-dominant to elicit specific HIV-1 immune responses that may have an impact on the HIV-1 reservoir.

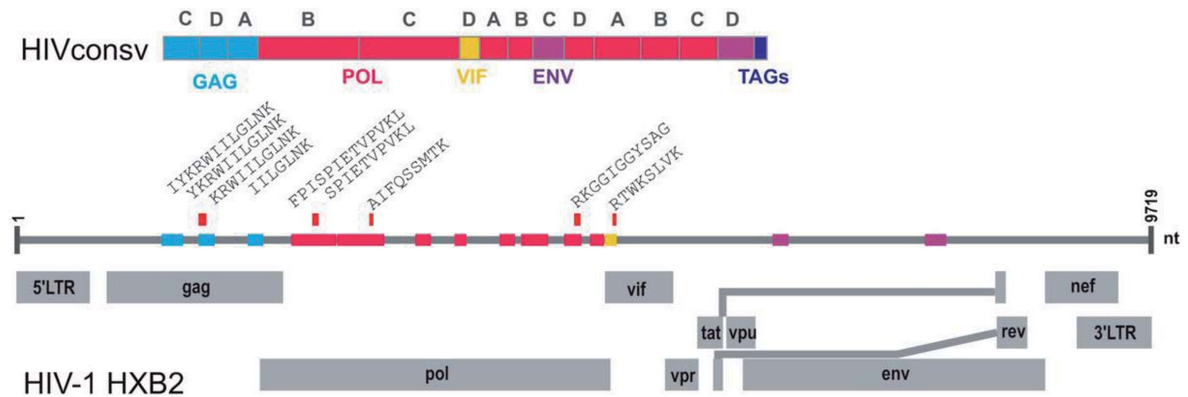


Figure 23. Schematic view of the HIVconsv immunogen, with the 14 conserved regions of HIV-1 that were combined in the immunogen represented as colored boxes and with the original HIV-1 region stated (Gag, Pol, Vif, and Env). Letters above the boxes (A, B, C, and D) indicate the clade of origin. The positions of eluted peptides identified in the HIVconsv immunogen are indicated by red bars. Genomic regions of the HXB2 strain are shown as grey rectangles. TAGs, epitope tag sequences; LTR, long terminal repeat; nt, nucleotides. Adapted from Ternette N. *et al.* (211).

3C.2. Material and Methods

3C.2.1. Patients

We conducted a randomized double-blind placebo-controlled study. This was approved by the Gene Therapy Advisory Committee (GTAC 165), the Medicines and Health Products Regulatory Agency (Eudract No. 2009-012662-31) and the Oxford University Hospitals NHS Trust. Written informed consent was obtained from all participants. Chronically HIV-infected adults aged 18-60 years who were virologically suppressed (<50 copies/ml) on combination ART and with $CD4^+$ cell counts >350 cells/µl were randomly assigned in a 4:1 ratio to receive vaccine or placebo in two groups of ten, the first group receiving an MVA.HIVconsv dose of $5.5x10^7$plaque-forming units (pfu) (low dose group) and the second, a dose of $2.2x10^8$ pfu (high dose group).

3C.2.2. Randomization and masking

Study participants were randomly assigned to receive vaccine or placebo according to the study protocol using a computer-generated block randomization scheme, which was generated by an independent statistician and held securely with restricted access.

3C.2.3. Vaccine and vaccination schedule

The MVA.HIVconsv vaccine has been described previously (210). Briefly, MVA.HIVconsv is composed by the immunogen HIVconsv vectored with the modified vaccinia Ankara viruse (MVA). HIVconsv comprises 14 highly conserved regions of the viral proteome, assembled end-to-end as a chimeric protein and balanced for representation of the major clades of HIV-1, A-D. It was manufactured and diluted in formulation buffer to $5x10^8$ pfu/ml by IDT Biologika GmbH, Germany. Formulation buffer was used as placebo. Vaccine and placebo vials were labeled by the manufacturer and stored at -80°C until use. The vaccine was thawed ≤30 minutes prior to administration and was given by intramuscular injection with a needle and syringe into the deltoid region of both arms (half of each dose per arm) on days 0, 28 and 84. Safety evaluations included reactogenicity symptoms recorded by the participants on diary cards following each vaccination, physical examination and monitoring of laboratory parameters. Blood samples were taken for

safety and immunogenicity evaluations according to the study protocol schema shown in Figure 24.

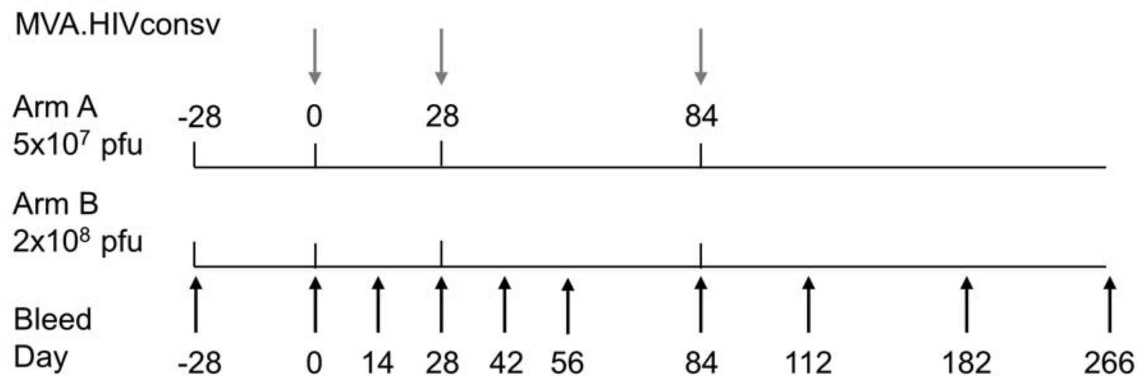


Figure 24. Study protocol. Volunteers were screened up to 28 days before vaccination and randomized on day 0 to receive either $5.5x10^7$ pfu MVA.HIVconsv or placebo (Arm A, subjects 1-10, 4:1 ratio) and either $2.2x10^8$ pfu MVA.HIVconsv or placebo (Arm B, subjects 11-20, 4:1 ratio), by intramuscular injection on days 0, 28 and 84. Blood was sampled on the days indicated.

3C.2.4. Peptides

HIVconsv peptides that span the HIVconsv immunogen (199 individual 15-17mer peptides overlapping by 11 amino acids) were pooled in a matrix format. The use of a peptide matrix enables deconvolution of the response and identification of individual peptide-specific responses using a two-step process (212). First, fresh PBMCs from each time-point were tested against all matrix peptide pools in an IFN-γ Elispot assay. Individual HIVconsv peptides from pools giving a positive response were then retested in a second IFN-γ Elispot assay using cryopreserved PBMCs. Responses were either confirmed as true positives or discounted. A 4-dimensional matrix was designed using 'Deconvolute This' (version 1.0 Roederer) and comprised 80 pools with 10 peptides per pool. 96-well plates containing working peptide pool stocks (4μg/ml) were prepared in batches and stored at -80°C until required; final peptide concentration in the Elispot assay was 2μg/ml.

3C.2.5. Ex vivo IFN-γ Elispot assay

IFN-γ Elispot assays were performed as described previously (213). The total HIVconsv specific response was defined as the sum of the magnitude of all individual HIVconsv peptides retested in the second-round assay that elicited a response greater than the mean plus 2 standard deviations (SD) of negative control well values (100 SFU/million PBMCs).

3C.2.6. Viral inhibition assay

The viral inhibition assay has been described in detail previously (214). CD8+ T cells were isolated from cryopreserved PBMCs by negative immunomagnetic selection ($CD8^+$ T cell isolation kit; Miltenyi Biotec), following the manufacturer's instructions, and used *ex vivo* without stimulation. The CCR5-tropic clade B laboratory-adapted HIV-1 isolate, BaL, was used for $CD4^+$ T cell super-infection at a multiplicity of infection of 0.01. $CD8^+$ T cell antiviral activity was reported as % inhibition.

3C.2.7. Quantification of HIV-1 proviral DNA in $CD4^+$ T cells

Lysed extracts from 10^6 $CD4^+$ T cells were used to measure total cell-associated HIV-1 DNA by ddPCR, as previously described in chapter 3A.

3C.2.8. Residual plasma viremia

To evaluate HIV-1 RNA below 50 copies/ml, plasma samples were ultracentrifuged and quantified using RT-qPCR, as previously described in chapter 3A.

3C.2.9. Statistical analysis

HIVconsv-specific T cell IFN-γ responses were analyzed over time and according to group allocation using linear mixed effects models (215,216). $CD8^+$ T cell viral inhibitory activity and total HIV-1 DNA were analyzed over time and according to group by non-parametric tests using GraphPad Prism software (version 5.0). Censored matched pairs of ultrasensitive viral load were analyzed using the paired Prentice-Wilcoxon test (MiniTab® v16.2.3).

3C.3. Results

3C.3.1. Study participants and vaccinations

Nineteen subjects (14 men and 5 women) were enrolled in the study. Enrolment was stopped prior to reaching the planned 20 subjects due to slow recruitment. Vaccine recipients and placebos did not differ significantly in age, $CD4^+$ cell count, $CD4^+$ cell nadir or duration of ART at baseline. Volunteer characteristics are summarized in Table 1. All volunteers received their vaccinations as scheduled, except for one vaccinee in the low dose group and one in the high dose group, who both withdrew after their second immunization (Fig 25).

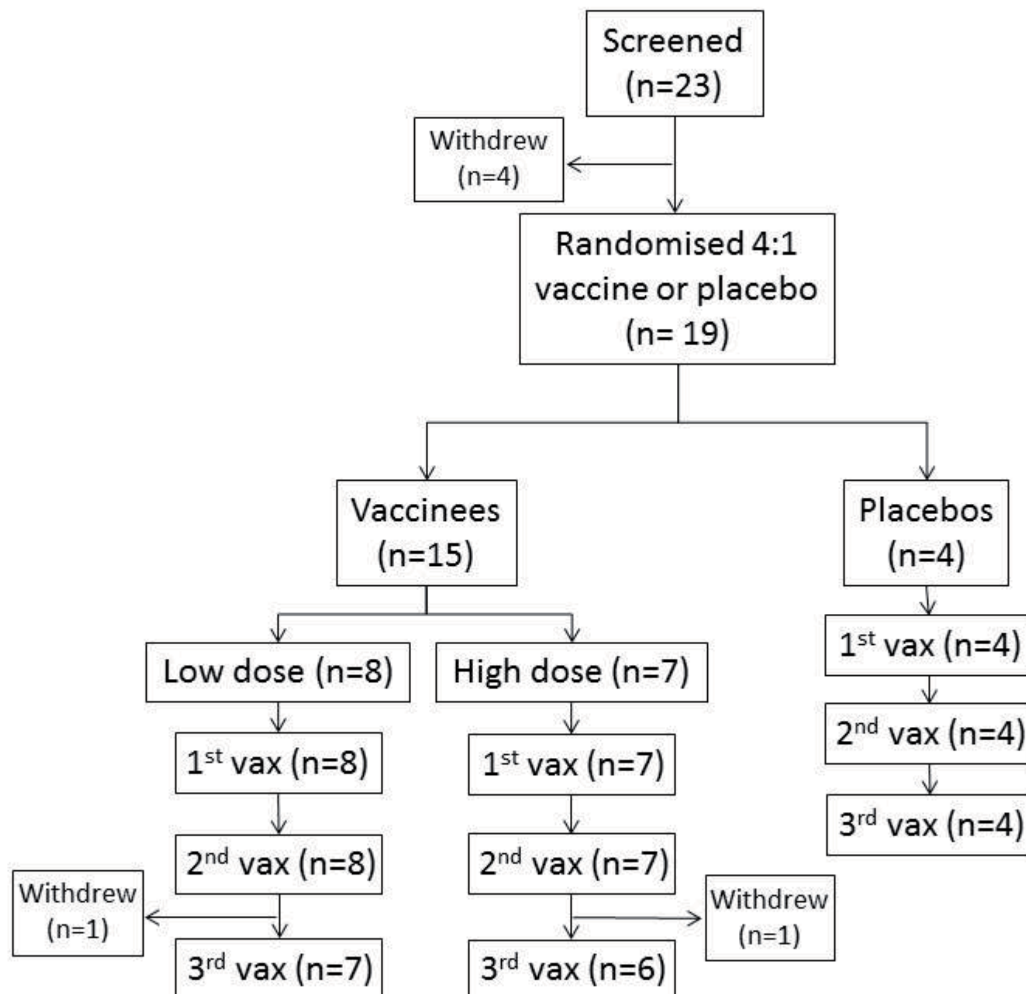


Figure 25. CONSORT flow diagram for recruitment and follow-up in the HIV-CORE 001 trial.

3C.3.2. Vaccine safety

Vaccination was safe and well tolerated. The majority (14/15) of vaccine recipients experienced mild (n = 10) or moderate (n = 4) pain at the injection site and 2/15 developed a mild local reaction. Nine vaccinees experienced mild to moderate malaise; two reported mild headache, and four reported 'chills' after vaccination. All of these reactions resolved spontaneously within 48 hours (Table 4). Mild pain, headache and moderate malaise were also reported by each of 3/4 placebo recipients. No subject experienced a severe local or systemic reaction. A total of 86 adverse events were reported during following (66 in the vaccinees and 20 in the placebos); of these, 51 were grade 1, 27 were grade 2, five were grade 3; in three, the grading scale was not applicable (Table 5). The grade 3 events occurring in vaccinees were all deemed to be unrelated to vaccination (n = 3, vomiting requiring hospitalization followed by development of a pulmonary abscess; pyelonephritis; gout). One placebo recipient developed Hodgkin's lymphoma. No unexpected or vaccine-related serious adverse events occurred during the trial.

All patients remained virologically suppressed (HIV-1 RNA <50 copies/ml) throughout the follow up, apart from three subjects who were found to have detectable viremia (69-1,404 copies/ml) on a single occasion, which was not confirmed on repeat testing. $CD4^+$ T-cell counts did not change significantly during follow up (p = 0.9, one-way ANOVA).

Table 4. Local and systemic reactions to MVA.HIVconsv (Table continued to the next page)

Local adverse event	**Number of vaccinees**			
Grade	*Mild*	*Moderate*	*Severe*	*Very severe*
Pain	10/15	4/15	0/15	0/15
Local reaction	2/15	0/15	0/15	0/15
Itch	0/15	0/15	0/15	0/15
Vaccinees without any local adverse event	1/15			
	Number of placebos			
Pain	2/4	0/4	0/4	0/4
Local reaction	0/4	0/4	0/4	0/4
Itch	0/4	0/4	0/4	0/4
Placebos without any local adverse event	2/4			

Table 4 (continued). Local and systemic reactions to MVA.HIVconsv

Systemic adverse event	**Number of vaccinees**			
Grade	*Mild*	*Moderate*	*Severe*	*Very severe*
Systemic	0/15	0/15	0/15	0/15
Chills	3/15	1/15	0/15	0/15
Malaise	2/15	7/15	0/15	0/15
Headache	2/15	0/15	0/15	0/15
Vaccinees without any local adverse event	5/15			
	Number of placebos			
Systemic	0/4	0/4	0/4	0/4
Chills	0/4	0/4	0/4	0/4
Malaise	1/4	1/4	0/4	0/4
Headache	1/4	0/4	0/4	0/4
Placebos without any local adverse event	1/4			

Table 5. Number and grade of adverse events according to relationship with vaccination

	Grade	Relationship				
		Unrelated	*Unlikely*	*Possible*	*Probable*	*Definite*
Vaccinees	1	20	16	0	1	0
	2	10	8	2	3	0
	3	4	0	0	0	0
	4	0	0	0	0	0
Placebos	1	10	4	0	0	0
	2	4	0	1	0	0
	3	1	0	0	0	0
	4	0	0	0	0	0

Grading not applicable to 2 unrelated adverse events (possible syphilis re-infection and pregnancy) therefore they are not included here.

3C.3.3. T cell responses to MVA.HIVconsv

IFN-γ Elispot assays with the 4-dimensional HIVconsv peptide matrix were performed using fresh PBMCs in all subjects at screening, day 0, day 14, day 56 and day 266 time-points, as a minimum. All deconvoluted matrix data up to day 56 were collated and individual peptide sets for re-testing were devised for each subject based on these data. The day 56 visit was chosen because this corresponded with the peak response attained in the majority of subjects after two MVA vaccinations in other therapeutic vaccine trials (213,217). The patient-specific peptide sets were then tested using cryopreserved PBMCs from each time-point in a single IFN-γ Elispot assay. Pre-vaccination responses to HIVconsv were detected in 16/19 subjects, with a median frequency of 798 (range 110-6,807) spot forming units (SFU) per million PBMCs. Following vaccination, the peak response was not

synchronized among the vaccinees, in contrast to previous studies of therapeutic vaccines encoding full-length HIV-1 proteins (213,218), and occurred between days 14 and 56. All 15 vaccinees had HIVconsv-specific responses above 100 SFU/million PBMCs at the peak of response and the median frequency was 1,790 (range 180-10,300) SFU/million PBMCs vs. 285 (range 110-6,807) SFU/million PBMCs at baseline. However, responses among placebos also showed variation over time (Fig 26). In view of the inter-subject variation in baseline responses, together with lack of synchronicity in responses to vaccination, linear mixed models were used to test for an interaction between HIVconsv responses over time and vaccination. The magnitude of responses increased significantly over time ($p = 0.001$) but differences between vaccinees and placebos were not statistically significant ($p = 0.48$). IFN-γ responses to an influenza/EBV/CMV peptide pool remained constant during the study period, indicating that the variability in HIVconsv responses was not due to technical issues such as variation in assay conditions (Fig 27). Pre- and post-vaccination responses to HIVconsv were distributed evenly across the immunogen and increased in breadth over time (Table 6), although the difference between vaccinees and placebos was statistically inseparable. There was a significant correlation between magnitudes of responses at baseline and at the peak of response in the vaccinees ($r = 0.63$, $p < 0.0001$; Fig 28) suggesting that MVA.HIVconsv vaccinations had boosted pre-existing responses.

$CD8^+$ T cell antiviral activity was measured in all trial participants at one pre- and 2 post-vaccination time-points, except for one of the placebo recipients for whom sample limitations permitted testing of two time-points only. The pre-vaccination median inhibitory response was 24% overall (3.5%, 42% and 22% in placebos, low dose and high dose vaccinees respectively) when measured at a $CD4^+/CD8^+$ T cell ratio of 1:1 ($p = 0.057$, Kruskall Wallis test). At the peak of response post-vaccination, median inhibition was 4.6%, 12.5% and 54% in placebos, low dose and high dose vaccinees respectively. In the high dose vaccinees there was a statistically significant increase in inhibition following vaccination with MVA.HIVconsv ($p = 0.004$, Friedman test; Fig 29), but no such trend was observed in the placebos or low dose vaccinees ($p = 0.19$ and 0.29 respectively).

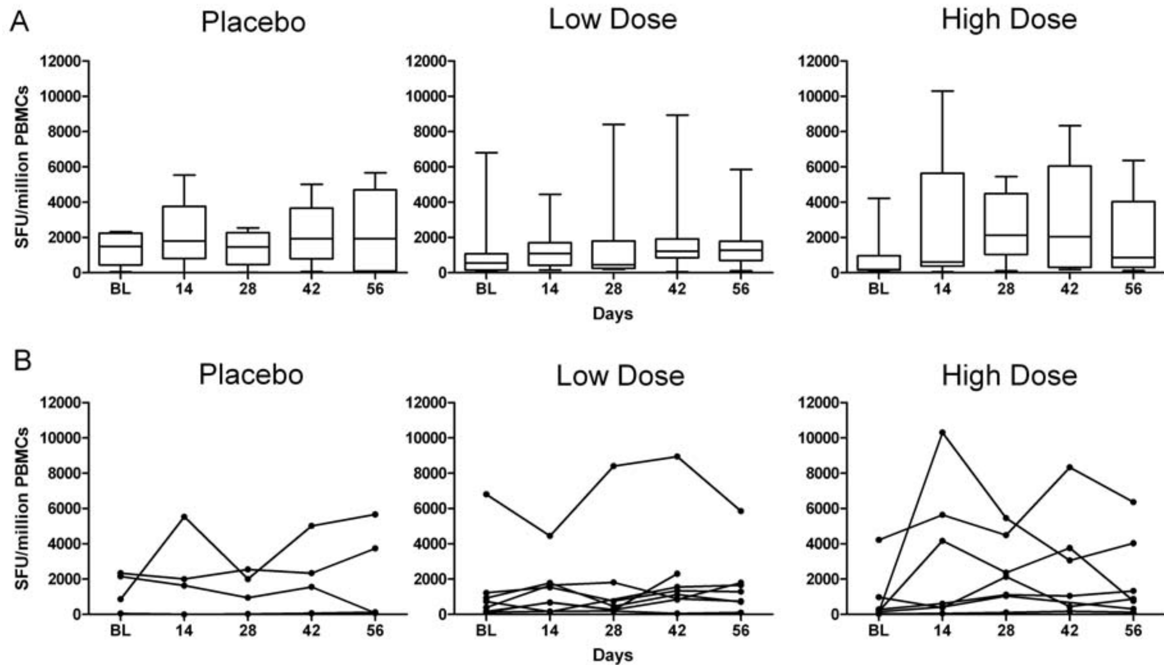


Figure 26. T cell immunogenicity of MVA.HIVconsv in volunteers over time. Total magnitude of T cell responses to the HIVconsv immunogen was assessed in ex vivo IFN-γ ELISPOT assays in (L to R) placebos, low dose vaccinees and high dose vaccinees shown as **(A)** box and whisker plot (median, IQR and range) and **(B)** line graphs (representing each subject). Total magnitude was calculated from the sum of all HIVconsv individual peptides tested in a confirmatory second round assay which gave a response above the mean plus 2* SD of negative control wells (100 SFU/million PBMCs, see Materials and Methods). BL - baseline.

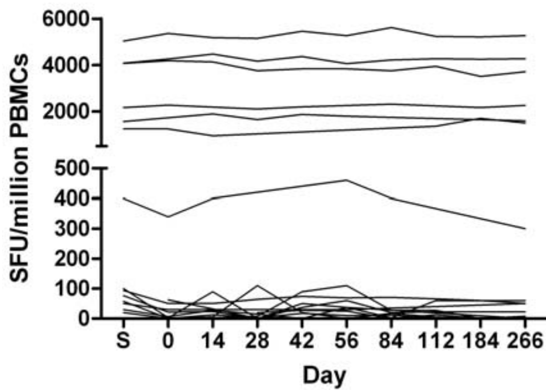


Figure 27. IFN-γ responses to an influenza/EBV/CMV peptide pool during follow-up. T cell responses to a pool of 22 influenza, EBV and CMV peptides restricted by common HLA class I alleles were measured in IFN-γ Elispot assays at each time-point throughout the study in all volunteers. S - screening.

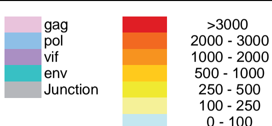
Table 6. Breadth of pre- and post-vaccination responses to HIVconsv. Location of confirmed positive peptides across the HIVconsv immunogen pre and post-vaccination (included if peptide was above 100 SFU/million PBMCs at any time-point after screening/day 0). Left-hand column shows peptide numbers according to antigenic origin: pink – gag; blue – pol; purple – vif; turquoise – env; grey - junctional peptide. Colour and number in boxes indicate magnitude of response. Patient numbers outlined with a black box indicate placebo volunteers.(Table continued to the next page)

HIVConsv		201		202		203		204		205		206		207		209		210		213		214		215		216		217		218		219		221		222		223	
Peptide	Sequence	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	MEEKAFSPEVIPMFT													163	225																								
2	AFSPEVIPMFTALSE													173	288											30	160												
5	LSEGATPQDLNTMLN																									1110	2800												
6	ATPQDLNTMLNTVGG																									2170	3100												
9	VGGHQAAMQMLKDTI																											410	280										
11	KQMLKDTINEEAAEWD													28	158					0	203																		
12	DTINEEAAEWDRIYK	0	130																																				
13	EEAAEWDRIYKRWII			53	110																							960	420			0	195						
15	IYKRWIILGLNKIVR																	1228	1537																				
16	WIILGLNKIVRMYSP																					350	500																
17	GLNKIVRMYSPVSIL															50	405					1720	2060			55	360					45	270						
22	PKEPFRDYVDRFARN															0	520					0	165																
23	FRDYVDRFARNCRAP	0	265																																				
24	VDRFARNCRAPRKKG													0	150																								
25	ARNCRAPRKKGCWKC	0	235																			0	370																
29	KEGHQMKDCTERQAN													0	120																								
30	KQMKDCTERQANFLGK															0	525																						
31	CTERQANFLGKIWPS																			63	463																		
36	MIGGIGGFIKVRQYD							75	150																														
37	IGGFIKVRQYDQILI																																						
38R	IKVRQYDQILIEICGKK																																						
39	KQYDQILIEICGHKAI							45	125																														
40	ILIEICGHKAIGTVL															255	485																						
44	GPTPVNIIGRNLLTQ																			53	103																		
47	LTQIGCTLNFPISPI															35	625													15	1070								
49	KNFPISPIETVPVKLK																			693	807																		
50	SPIETVPVKLKPGMD																			1003	1067																		
52	KLKPGMDGPKVKQWP	0	175																																				
53	GMDGPKVKQWPLTEE																											350	350										
54	PKVKQWPLTEEKIKA																											230	180										
60	KEGKISKIGPENPYN																							45	1010														
64	PVFAIKKKDSTKWRK																															55	135						
68	VDFRELNKRTQDFWE																					0	137																
72	KQLGIPHPAGLKKKKS															0	505					0	125																
74	GLKKKKSVTVLDVGD	0	105																													0	205						
75	KKSVTVLDVGDAYFS															0	725			1233	1577																		
76	TVLDVGDAYFSVPLD																			1263	1293																		
78	YFSVPLDEGFRKYTA																			0	1203																		
79	PLDEGFRKYTAFTIP																			0	723									5	1400								
80	GFRKYTAFTIPSINN																													20	1220								
81	YTAFTIPSINNETPG															0	465																						
82	TIPSINNETPGIRYQ	0	240									0	440											40	545														
83	INNETPGIRYQYNVL											128	1100													860	1910					45	245						
84	TPGIRYQYNVLPQGW							65	180																														

Antigenic origin	Response magnitude (SFU/million PBMCs)
gag	>3000
pol	2000 - 3000
vif	1000 - 2000
env	500 - 1000
Junction	250 - 500
	100 - 250
	0 - 100

Table 6 (continued). Breadth of pre- and post-vaccination responses to HIVconsv. Location of confirmed positive peptides across the HIVconsv immunogen pre and post-vaccination (included if peptide was above 100 SFU/million PBMCs at any time-point after screening/day 0). Left-hand column shows peptide numbers according to antigenic origin: pink – gag; blue – pol; purple – vif; turquoise – env; grey - junctional peptide. Colour and number in boxes indicate magnitude of response. Patient numbers outlined with a black box indicate placebo volunteers.

HIVConsv		201		202		203		204		205		206		207		209		210		213		214		215		216		217		218		219		221		222		223	
Peptide	Sequence	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
85	RYQYNVLPQGWKGSP							35	220																														
87	KQGWKGSPAIFQSSMT																																			380	1575		
88	GSPAIFQSSMTKILE																																			595	1820		
91	ILEPFRAQNPEIVIY	0	175																	483	392																		
92R	FRAQNPEIVIYQYMDKK							25	140											823	867																		
93	KNPEIVIYQYMDDLYV									50	125			98	245					833	793	260	410									65	120					30	180
94	VIYQYMDDLYVGSDL	0	225											118	250					313	882											15	240						
97	SDLEIGQHRMENRWQ													18	105																								
98	IGQHRMENRWQVMIV	0	230																																				
105	LVKHHLTEEAELELA													108	125																								
110	LKDPVHGVYYDPSKD							25	120																														
116	ATWIPEWEFVNTPPL																													10	1840								
117	PEWEFVNTPPLVKLW																													20	1935								
124	WKNDMVDQMHEDIIS															0	425																						
132	AHKGIGGNEQVDKLV													128	53																								
139	DKAQAKEIVASCDKC																			43	417																		
143	LKGEAMHGQVDCSPG	0	255																																				
148	CTHLEGKVILVAVHV															0	785																						
153	EAEVIPAETGQETAY					43	258																							5	285								
154	IPAETGQETAYFLLK											0	150																	5	175								
158	AMNKELKKIIGQVRD	0	330																	0	167																		
159	ELKKIIGQVRDQAEH																													35	460								
160	IIGQVRDQAEHLKTA	0	175																																				
161	VRDQAEHLKTAVQMA																							25	1010														
162	AEHLKTAVQMAVFIH			73	100			35	280															60	1895			400	450										
163	KTAVQMAVFIHNFKR	0	218																	0	217							70	200										
164	VQMAVFIHNFKRKGGI							35	240																														
165	FIHNFKRKGGIGGYS													78	140																								
169	GERIWKGPAKLLWKG													28	115															30	1675								
170	WKGPAKLLWKGEGAV																													0	450								
171	AKLLWKGEGAVVIQD					53	1008																																
172	WKGEGAVVIQDNSDI					13	173					0	100																										
173	GAVVIQDNSDIKVVP																																						
174	IQDNSDIKVVPRRKA							0	100																							60	135						
177	RKAKIIRDYGKQMAG																																	30	300				
178	IIRDYGKQMAGADCV																																	10	130				
185R	MTLTVQARQLLSGIVKK															20	685																						
187	RQLLSGIVQQQNNLLR															0	515																						
189	VQQQNNLLRAIEAQQHL							75	430																														
190	LLRAIEAQQHLLQLT	38	145					115	115							255	533																						
192	QHLLQLTVWGIKQAC															245	525																						



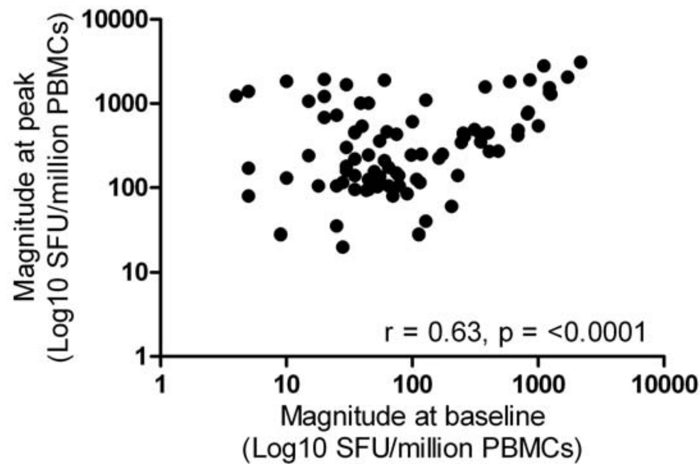


Figure 28. MVA.HIVconsv vaccination boosted pre-existing responses. Correlation between magnitude of IFN-γ responses to HIVconsv peptides at baseline and at the peak post-vaccination. Data points show individual HIVconsv peptides retested in second round assays with a response above 100 SFU/million PBMCs. Strength of the association was assessed using the Pearson correlation test.

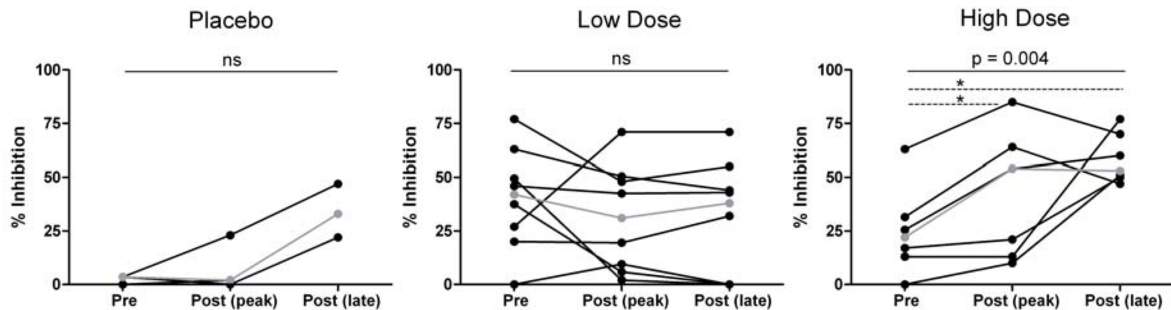


Figure 29. CD8+ T antiviral activity is increased following vaccination with a higher dose of MVA.HIVconsv. CD8+ T cell-mediated inhibition of HIV-1_{Bal} in autologous CD4+ T cells (CD8+/CD4+ cell ratio = 1:1) was measured in all 19 trial participants at baseline (Screening or day 0), post-vaccination, concurrent with the peak response in the IFN-γ Elispot assay, and at a late post-vaccination time-point (day 182 or day 266) except one placebo recipient (pre-vaccination and peak only). This individual was therefore excluded from the statistical analysis. Grey line indicates median response. Asterisks indicate statistically significant differences in multiple comparisons analysis.

3C.3.4. Effect of MVA.HIVconsv vaccinations on the HIV-1 reservoir

HIV-1 DNA was detected in all study subjects at all time-points tested (medians 1,065, 1,014 and 1,182 copies/10^6 $CD4^+$ T cells at days 0, 42 and 266 respectively). Copies of HIV-1 DNA/10^6 $CD4^+$ T cells at baseline did not correlate with $CD4^+$ T cell nadir (r = 0.42, p = 0.15), $CD4^+$ cell count (r = -0.23, p = 0.33), interval between diagnosis and initiation of treatment (r = 0.17, p = 0.54) or duration of ART. There was no change from baseline in the size of the reservoir at day 42 or day 266 post-vaccination in the vaccinees as a whole (medians 1,032, 944 and 960 copies HIV-1 DNA/10^6 $CD4^+$ T cells, p = 0.77; Friedman test) or placebos (medians 1,652, 2,058 and 1,351 copies HIV-1 DNA/10^6 $CD4^+$ T cells, p = 0.65; Friedman test). Stratification of vaccinees into low and high dose groups also did not

reveal any significant changes post-vaccination (days 0, 42 and 266 medians for low dose 1,074, 1,260 and 1,272 copies HIV-1 DNA/10^6 $CD4^+$ T cells, $p = 0.65$; and high dose 827, 721 and 577 copies HIV-1 DNA/10^6 $CD4^+$ T cells, $p = 0.23$; Friedman test) (Figure 30A). When HIV-1 DNA was expressed as a ratio of day 42 or day 266 to baseline values, this confirmed that there was no significant change during the study (Figure 30B).

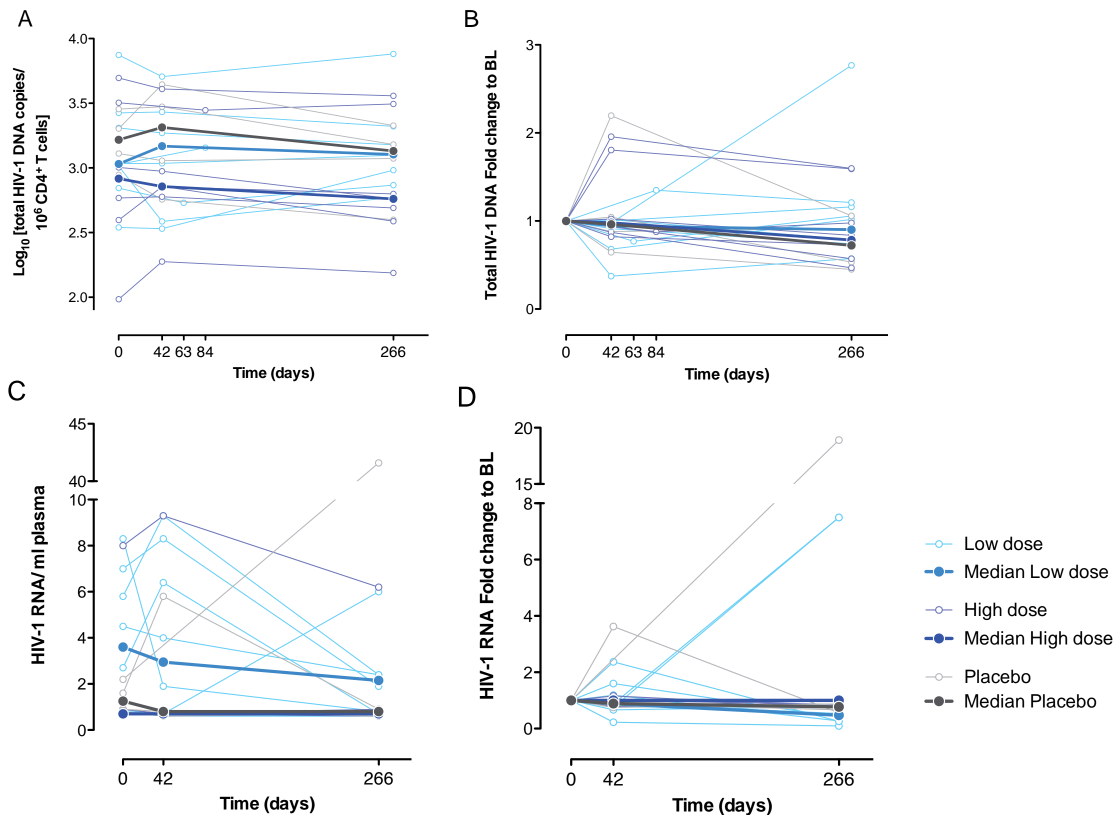


Figure 30. Quantification of the HIV-1 reservoir following vaccination with MVA.HIVconsv. **(A)** Total HIV-1 DNA (copies/million $CD4^+$ T cells) was measured by quantitative ddPCR in trial participants at days 0, 42* and 266. **(B)** Fold change of total HIV-1 DNA to day 0. **(C)** Ultrasensitive viral load (HIV-1 RNA/ml plasma) was measured by Abbott Real-Time HIV-1 assay at days 0, 42 and 266. **(D)** Fold change of ultrasensitive viral load to day 0. *In three volunteers total HIV-1 DNA was measured at day 63 or 84 rather than day 42 due to sample availability, and there was no plasma sample at day 42 for one patient of the placebo group. Dark lines indicate median values for each group and light lines indicate individual values.

Although we observed no variation in the total HIV-1 reservoir size, we performed an ultrasensitive viral load assay to analyze whether MVA.HIVconsv affected the residual plasma viremia. We detected residual plasma viremia in 47% of the patients, with a range limit of detection (LOD) of 0.6-0.9 copies HIV-1 RNA/ml of plasma. We observed no changes from baseline in the ultrasensitive viral load at days 42 or 266 post-vaccination in any group (low dose, $p = 0.68$ and 0.12 respectively; high dose, $p = 0.32$ and 0.32 respectively; placebo, $p = 0.32$ and 0.53

respectively; paired Prentice-Wilcoxon test; Figure 30C-D). Thus, MVA.HIVconsv did not affect the HIV-1 reservoir size nor the residual viremia, either short- or long-term after vaccination.

3C.4. Discussion

We investigated the immunogenicity of a conserved region immunogen, HIVconsv, tested for the first time in HIV-1 infected individuals on ART. A homologous prime-boost regimen of three MVA.HIVconsv vaccinations was safe and well tolerated. Interferon-γ T-cell responses to conserved epitopes, many of which are subdominant in natural infection, increased after vaccination. However, the magnitude of the change was not significantly greater in vaccinees than placebos, due to an unanticipated level of variation in responses over time in the latter. By contrast, responses to non-HIV-1 antigens remained constant during the study period in vaccinees and placebos. This highlighted the natural intra-individual variation in HIV-specific T cell responses in long-term ART-treated patients, which has not been extensively reported before (219). Of note, however, we identified a large number of responses to HIVconsv peptides that were targeted to new or rare viral epitopes, since they were either absent from the Los Alamos National Laboratory HIV Immunology Database or were restricted by HLA alleles not shared with the patients. While these epitopes have not yet been optimally defined, our data suggest that MVA.HIVconsv had primed T cell responses to epitopes that are infrequently recognized in natural infection. These could be attractive targets for more potent immunotherapeutic approaches to purge reactivated $CD4^+$ T cells in which ‘CTL-resistant’ viruses predominate (145). We have observed that heterologous vaccination regimens comprising a replication-defective chimpanzee adenovirus vector prime followed by MVA boost elicited high frequencies of HIVconsv-specific T cells in healthy HIV-uninfected individuals (151). Preliminary data indicate that this approach is similarly immunogenic in HIV-positive patients initiating ART during primary infection (220).

In contrast to IFN-γ responses, the antiviral inhibitory capacity of $CD8^+$ T cells was enhanced by vaccination with MVA.HIVconsv in the subjects receiving the higher dose (2.2×10^8 pfu). The inhibitory activity was still below levels we have observed in individuals who spontaneously control HIV-1. Nevertheless, this does suggest that HIV-specific $CD8^+$ T cell function can be enhanced by vaccination, even in chronic infection (214,221). Possible explanations for the modest responses in the patients studied here are: (i) irreversibly compromised $CD4^+$ T cell helper function at the time of ART initiation (CD4 nadir was <200 cells/μl in the majority);

(ii) limited potency of a homologous MVA vector vaccine regimen and (iii) insufficient coverage by the HIVconsv immunogen of viral epitopes that are crucial to its fitness. The first two of these issues are being addressed in an ongoing clinical trial (220). Regarding the immunogen design, responses to conserved regions of the HIV-1 proteome have been associated with control of virus replication (222,223). However, we have shown that potent $CD8^+$ T cell inhibitory activity is better explained by targeting of specific vulnerable regions of the viral proteome that were not predicted by conservation algorithms alone (221), likely because sequence conservation does not correlate strongly with genetic fragility (224,225). Inclusion of more vulnerable viral epitopes in the immunogen may be necessary to optimize boosting of $CD8^+$ T cell responses that are the most relevant to virus control (226).

We reasoned that therapeutic vaccination with MVA.HIVconsv could reduce the size of reservoir through direct and indirect but non-mutually exclusive mechanisms. First, $CD4^+$ T cell activation by MVA-derived antigens could theoretically induce reactivation of latently infected cells (227), via the TLR1/2 signaling pathway for example (228,229). Secondly, re-stimulation of HIV-1 specific $CD8^+$ T cell populations that may have contracted as a consequence of virological suppression could accelerate the clearance of $CD4^+$ T cells that express cognate epitopes at sufficient levels for immune recognition, whether reactivated or resting (230). In this study, HIV-1 reservoir size and residual plasma viremia, as measured by total HIV-1 DNA copies in $CD4^+$ T cells and ultrasensitive viral load respectively, did not change significantly after vaccination. This indicates that recombinant MVA vaccination alone does not reactivate latent HIV-1 nor induce T cell responses of sufficient potency to perturb the reservoir. Consistent with this, in another study investigating the effects of an MVA-vectored vaccine encoding HIV-1 Gag, vaccination led to a modest increase in the frequency of Gag-specific T cells and a transient delay (4 weeks) in viral rebound after interruption of ART but no sustained effect on the viral reservoir (139). A similar approach involving a DNA / recombinant human adenovirus type 5 prime-boost HIV-1 vaccination strategy in chronically infected patients receiving intensified ART also failed to increase HIV-1 expression or reduce the size of the reservoir (231). These findings indicate that both arms of a ‘shock and kill’ strategy need to be optimized, which represents a significant

challenge. First, latency-reversing agents that induce adequate HIV-1 antigen expression for triggering of cytolysis while avoiding global T cell activation have yet to be identified. Second, reactivation must be synergized with killing by vaccine-induced T cells. Third, T cells must recognize epitopes generated by reactivated cells, which may be different from the sequences to which they were primed. The window of opportunity for the latter may be limited to a few hours (232). Furthermore, we have recently shown, using tandem mass spectrometry, that the HLA class I-associated HIV-1 peptidome on primary $CD4^+$ T cells includes a substantial proportion of epitopes that had not been described previously (233). These observations highlight the need for improved understanding of the kinetics, breadth and density of viral epitope expression on reactivated primary $CD4^+$ T cells.

This study is, to our knowledge, the first to investigate the relationship between the HIV-1 reservoir size and $CD8^+$ T cell viral inhibitory activity in chronic ART-treated patients. We did not observe a correlation between these parameters before or after vaccination. As the patients in our study had been infected for a median 6 years, this could reflect the stability of the reservoir at this stage of infection and the limited capacity of the vaccine to enhance $CD8^+$ T cell viral inhibitory activity. We have previously reported that a cut-off of ≥85% in our viral inhibition assay distinguished individuals with natural control of HIV-1 viremia (<2000 copies/ml) from non-controllers (221). Such individuals typically have smaller viral reservoirs than progressors (234,235). Further analysis of $CD8^+$ T cell inhibition in therapeutic HIV-1 vaccine trials may help to identify possible immunological surrogates of vaccine efficacy in the context of HIV-1 eradication strategies.

In summary, our study highlights the need for further optimization of therapeutic vaccines for inclusion in HIV-1 eradication strategies. In addition, the high degree of variability in HIV-specific IFN-γ T cell responses during long-term ART needs to be considered in clinical trial design. Testing of candidate immunological endpoints that are predictive of changes in the viral reservoir is a critical component of such studies.

CHAPTER 3D. Cellular therapy

This chapter correspond to the manuscript:

CCR5 Δ32 homozygous cord blood allogeneic transplantation in a patient with HIV: a case report

Rafael F. Duarte, María Salgado, Isabel Sánchez-Ortega, Montserrat Arnan, Carmen Canals, Eva Domingo-Domenech, Alberto Fernández-de-Sevilla, Eva González-Barca, Sara Morón-López, Nuria Nogues, Beatriz Patiño, Maria Carmen Puertas, Bonaventura Clotet, Lawrence D. Petz, Sergio Querol, Javier Martinez-Picado

Lancet HIV, 2015

3B.0. Presentation

This chapter evaluates the role of cord blood stem cell transplantation with the a 32 bp deletion in *CCR5* gene of the donor cells, as a cellular gene approach to cure HIV-1 infection, due to the complete elimination of the latently infected cells and the reconstitution of an HIV-1 resistant immune system.

3D.1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) from a homozygous *CCR5* delta32 unrelated adult donor by Hütter *et al*. provides the only evidence to date of long-term control of HIV-1 infection (158,236). Viral rebound following HSCT with heterozygous or wild-type variants makes a compelling argument in favor of *CCR5* Δ32/Δ32 allogeneic reconstitution as the key mechanism driving resistance to infection in the Berlin patient (159,237–239). Unfortunately, the search for “patient number two” remained unsuccessful for many years (240), until a recent such case by Kordelas *et al.* showed that in the presence of CXCR4-tropic HIV-1, *CCR5*-knockout strategies may fail to control HIV-1 infection (241,242). Broader investigation of the conditions for this proof-of-concept strategy to benefit other HIV-1 infected individuals requires wider availability of *CCR5* Δ32/Δ32 hematopoietic stem cells, which the low prevalence of the genotype (less than 1% in Caucasians and lower in other ethnic groups), and the stringent human leukocyte antigen (HLA) matching criteria make it unfeasible to obtain from conventional adult HSCT donors (160,161,243).

Hematopoietic stem cells from cord blood (CB) allow more permissive HLA-compatibility (244), leading recent efforts in this field towards the development of readily available inventories of *CCR5*-screened Δ32/Δ32 units as a potential platform for allogeneic HSCT in HIV-infected individuals (245,246). Here, we describe a first reported case of allogeneic HSCT using *CCR5* Δ32 homozygous CB cells in a patient with HIV-1 infection and a diffuse large B-cell lymphoma (DLBCL).

3D.2. Material and Methods

3D.2.1. Chimerism analysis

Postransplant chimerism was analyzed on full peripheral blood mononuclear cells and on immunomagnetically selected $CD3^+$ and $CD15^+$ cells with microsatellite markers fluorescent PCR (AmpFLSTR® Identifiler® Plus, Applied Biosystems), and indel markers high-resolution real-time PCR (Mentype® DIPquant, Biotype) for selected samples (247).

3D.2.2. CCR5 genotyping and viral tropism assay

CCR5 genotyping was analyzed as previously described (248). HIV-1 full envelope was amplified from the patient and recombinant viruses were made in MT-4 cells (in combination with a NL43-Δenv plasmid). U87-CD4-CCR5 and U87-CD4-CXCR4 cells were infected with both, Env-recombinant virus and two different viral isolates from the patient, and tropism was determined by HIV-1 replication (HIV-1 $p24^{gag}$ ELISA). Additionally, the patient's virus envelope was sequenced and tropism was predicted using Geno2Pheno software (249).

3D.2.3. Virus Isolation and Sequence Analysis

Isolation of replication-competent virus and full HIV-1 genome amplification from bulk $CD4^+$ T-cells were performed as previously described (250). Total $CD4^+$ and resting $CD4^+$ T-cells were isolated by negative immunomagnetic selection ($CD4^+$ T cell isolation kit; Miltenyi Biotec) and cultured following qVOA protocol described in chapter 3A. Two different replication-competent isolates were obtained from total $CD4^+$ T-cells. Resistance to ART was predicted from full viral genome sequences from the patient $CD4^+$ T cells (251).

3D.2.4. Reservoir parameters

HIV-1 reservoir analyses included cell-associated HIV-1 DNA and RNA, and replication-competent virus were performed as described in chapter 3A. Residual plasma viremia was quantified as previously described in chapter 3B.

3D.2.5. Semiquantitative PCR based detection

Semiquantitative PCR-based detection of Gag proviral DNA consisted of a first PCR using the primers forward 5'-GCGAGAGCGTCAGTATTAAGC-3', and re-

verse 5'-TCTTTATCTAAGGGAACTGAAAAATATGCATC-3'. This was followed by a nested-PCR using the primers forward 5'-GGGAAAAAATTCGGTTAAGGCC-3', and reverse 5'-CGAGGGGTCGTTGCCAAAGA-3'. For both PCRs, high fidelity PCR buffer (Invitrogen) was mixed with magnesium sulfate solution (2 mM; Invitrogen), dNTPs Mix (0.2 mM; Invitrogen), the corresponding primers of each PCR (0.4 µM/each; Integrated DNA Technologies), Platinum® Taq DNA polymerase high fidelity (0.04 U/µl; Invitrogen) and 2.5 µl of cell lysate (equivalent to 125,000 cells) in a total volume of 25 µl. HIV-1 DNA was amplified using the Gene Amp® PCR System 2700 (Applied Biosystems) (initial denaturation: 94ºC, 3'; 34 cycles: 94ºC, 30"; 50ºC, 30", and 68ºC, 1' 45"; final extension: 68ºC, 5'; hold: 4ºC). The PCRs of 10-fold dilution of cell-associated HIV-1 DNA were performed in ten replicas. Amplification was analyzed by agarose gel electrophoresis (1%) and expressed as percentage of positive Gag PCR amplification per total PCR wells.

3D.2.6. Immune activation and Proliferation

PHA-treated PBMCs (5 µg/ml for three days) were tested for T-cell surface expression of activation markers (CD25, CD69 and HLA-DR) and for proliferation using an LSRII flow cytometer (Becton Dickinson), and the analysis was carried out using FlowJo software 9.2.3v (Tree Star). Data are presented as percentage of marker expression and loss of membrane CFDA expression (Vybrant® Cell Tracer Kit; Life Technologies) in $CD4^+$ T-cells before and after PHA-activation.

3D.2.7. Cell Infectivity Assay

Viral fitness was analyzed as described previously (252). $CD4^+$ T-cells from the patient and from two healthy donors were activated for three days as above and then infected by spinoculation (centrifugal inoculation at 1200xg for two hours) with the patient's primary viral isolates, or with laboratory viral strains $HIV\text{-}1_{NFN\text{-}SX}$ (CCR5-tropic) and $HIV\text{-}1_{NL4\text{-}3}$ (CXCR4-tropic). Supernatants were sampled over the course of seven days. Viral replication was quantified using HIV-1 $p24^{gag}$ ELISA.

3D.3. Results

3D.3.1 Case report

A 36 year-old man with HIV-1 infection known from September 2009 was referred to our hospital in March 2012 with a recent diagnosis of diffuse large B-cell lymphoma (DLBCL). At the onset of lymphoma, the patient was on effective antiretroviral treatment (ART) with suppressed plasma viremia (Figure 31), $CD4^+$ T-cell count of 360 per cubic millimeter and no illnesses associated with the acquired immunodeficiency syndrome. He presented with a large abdominal mass expanding from the left kidney to the iliac lymph nodes and additional paraaortic and retrocrural involvement (stage II-A). At the onset of DLBCL, the patient's $CD4^+$ T-cell count was 360 per cubic millimeter, and HIV-1 RNA copies <50 per milliliter. Prior to allogeneic HSCT, the patient received several lines of chemotherapy, including six courses of CHOP-R, three courses of ESHAP-R, an autologous HSCT, three courses of GEMOX, and local abdominal radiotherapy. At the time of allogeneic HSCT the patient presented a refractory lesion in the left psoas, no B symptoms, and had good clinical performance with adequate cardiac and pulmonary pre-transplant evaluations.

No HLA-matched sibling donors were available. Following approval by the Spanish Hematopoietic Donor Registry (REDMO), a directed search identified two compatible *CCR5* Δ32/Δ32 cord blood (CB) units (StemCyte; Table 7) (246). A dual allogeneic HSCT was performed following myeloablative conditioning with fludarabine 120 mg/kg, busulfan 12 mg/kg, cyclophosphamide 100 mg/kg and thymoglobuline 4 mg/kg, graft-versus-host disease (GVHD) prophylaxis with cyclosporine A plus short-course corticosteroids, and combining the single CB unit with higher cellularity (CBU#1) and purified CD34+ cells from a haploidentical sibling, as previously described (253–255). Blood and/or tissue samples were obtained before and weekly after HSCT to monitor HIV-1 infection. ART continued during the procedure (Figure 31). The patient provided informed consent and the institutional review board approved the transplant and investigational protocol (CEIC Bellvitge: 2013-023449).

Early transplant complications, including cyclosporine-related toxicity, hepatic venoocclusive disease and cytomegalovirus reactivation, all resolved with appro-

priate treatment. Neutrophil engraftment, 100% haploidentical donor-derived, occurred on day +11. However, donor switch from haploidentical to CB-origin did not follow as expected (Figure 32) (253–255). High-resolution chimerism analysis detected low level CB-derived hematopoiesis below 5% for up to seven weeks after transplant. Post-thawing analysis of CBU#1 showed a mild reduction in cell content and very poor clonogenic capacity (Table 8). CBU#2 was subsequently infused on day +52 following 4 mg/kg thymoglobuline. Within three weeks from CBU#2 infusion, the chimerism from CBU#1 increased very rapidly to 20% on day +59, 83% on day +66 and 100% by day +73. In this process, the patient's hematopoiesis, including $CD4^+$ T-lymphocyte subset analysis, changed from homozygous wild-type *CCR5* (recipient) to heterozygous wild-type/Δ32 (haploidentical sibling), and finally became homozygous for *CCR5* Δ32 (Figure 32). He did not develop any symptoms or signs of GVHD. Regretfully, our patient developed an aggressive progression of DLBCL. Following very rapid clinical deterioration, he passed away from disease progression three months after transplant.

Table 7. Characteristics of the patient and donors

	Recipient	CBU1	CBU2	TPHD
Age (years) or year of collection	37	2008	2008	41
Sex	Male	Male	Male	Male
Blood group	O positive	O positive	A positive	A negative
CCR5 genotype	WT,WT	Δ32,Δ32	Δ32,Δ32	WT,Δ32
HLA typing*				
HLA A	01:01, 68:01	01:01, 03:01	01:01, 02:XX	11, 68
HLA B	08:01, 07:02	08:01, 07:02	08:01, 07:02	35, 07
HLA DR	03:01, 13:01	03:01, 14:01	03:01, 14:01	01, 13

CBU: cord blood unit; TPHD: third-party haploidentical donor; WT: wild type; HLA: human leucocyte antigens
* Recipient had negative anti-HLA antibodies

Table 8. Cell products content

	CBU1 controls before HSCT	CBU1 after thawing[a]	CBU2 controls before HSCT	CBU2 after thawing[a]	TPHD
CD34 ($\times 10^5$/kg)	0.7	0.52	0.73	0.22	40[b]
TNC ($\times 10^7$/kg)	2.53	2.05	1.98	1.64	–
CFU-GM ($\times 10^4$/kg)	1.02[c]	0.11	0.12[c]	NG	–
Total CFU ($\times 10^4$/kg)	2.13[c]	0.21	0.42[c]	NG	–

CBU: cord blood unit; HSCT: hematopoietic stem cell transplantation; CFU: colony forming units; GM: granulocyte-macrophages; NG: no growth; TNC: total nucleated cells; TPHD: third-party haploidentical donor
[a] Subsequent quality control results in the final cell product after thawing the CBU for infusion
[b] Purified by cell positive selection to include fewer than 10,000 $CD3^+$ T cells per kg patient's bodyweight
[c] Quality controls were done at the transplant center's processing laboratory before transplantation in aliquot samples from segments attached to the CBU2 when data were not available in the original report from the cord blood bank

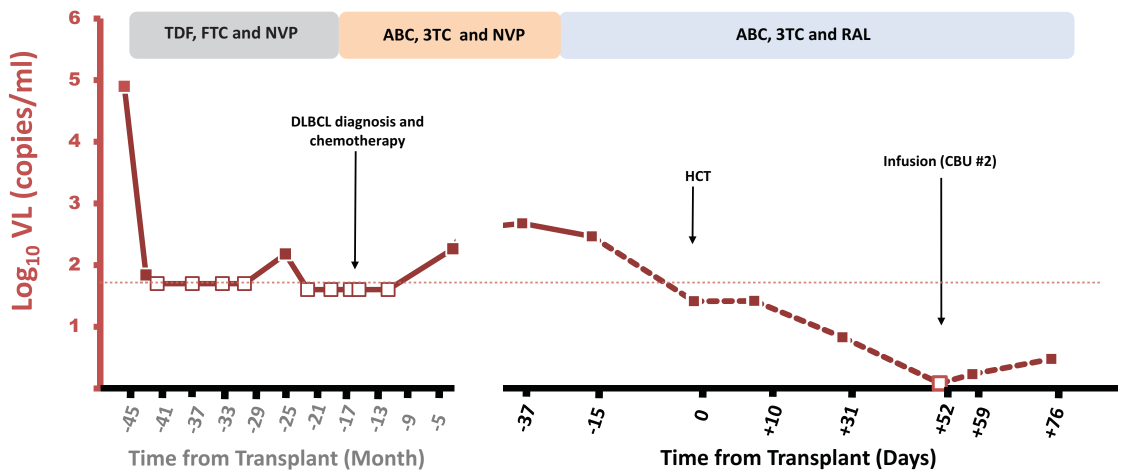


Figure 31. Clinical course including evolution of HIV-1 plasma viremia. Continuous lines represent viral loads measured with standard clinical tests before allogeneic transplantation, and dotted lines represent ultrasensitive viral loads (single copy assay) during the transplant process. Open symbols represent undetectable viral load values (<50 and <1 copies/ml, respectively). Antiretroviral therapy combined, as described, the following drugs at various periods of the clinical course: emtricitabine (FTC) 200 mg qd, tenofovir (TDF) 300 mg qd, nevirapine (NVP) 200 mg bid, lamivudine (3TC) 150 mg bid, abacavir (ABC) 300 mg bid, raltegravir (RAL) 400 mg bid. DLBCL: diffuse large B-cell lymphoma. HSCT hematopoietic stem cell transplantation. CBU: cord blood unit.

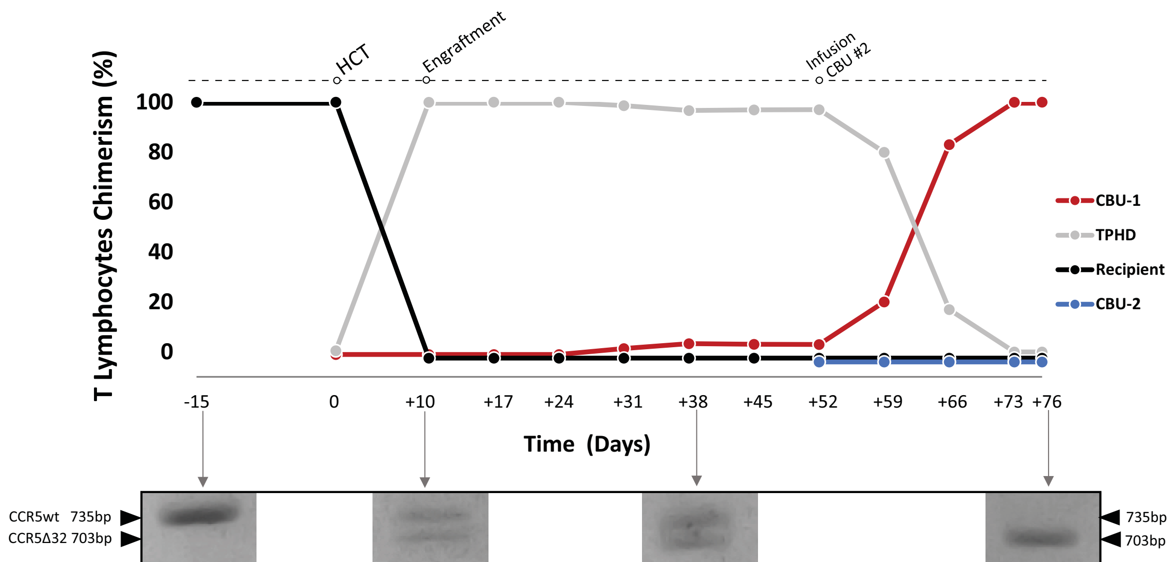


Figure 32. Postransplant chimerism kinetics showing recipient's, third party haploidentical donor's (TPHD), and CBUs' origin. Genotyping patterns of *CCR5* alleles are lined up below showing shift from *CCR5* wild-type (735bp, recipient's cells) to homozygous delta32 *CCR5* (703bp, CBU#1's cells). Intermediate samples from the TPHD show two bands (735+703bp), in keeping with his delta32 *CCR5* heterozygous genotype (see Table 5).

3D.3.2. Viral Characteristics and Tropism

Patient CD4^{+} T-cells full viral genome amplification showed no major deletions or mutations in the viral sequence, suggesting infection with a full pathogenic virus. Genotypic analysis showed HIV-1 to be B-subtype, no resistance mutations at the time of HSCT, and to be CCR5-tropic by Geno2Pheno bioinformatics (false positive rate 37.7%) (249–251). Additionally, we constructed a recombinant virus

carrying the patient's primary viral envelope gene sequences, obtained from bulk PCR, thus representing the viral quasiespecies present in the patient's T-cells. This recombinant virus and two other independent viral culture isolates were used for phenotypic tropism determination. All three viruses were able to replicate in U87-CCR5 cells but not in U87-CXCR4 cells (Figure 33), confirming that residual CXCR4-tropic virus was very unlikely, and that the patient's HIV-1 was CCR5-tropic by genotypic and phenotypic analyses.

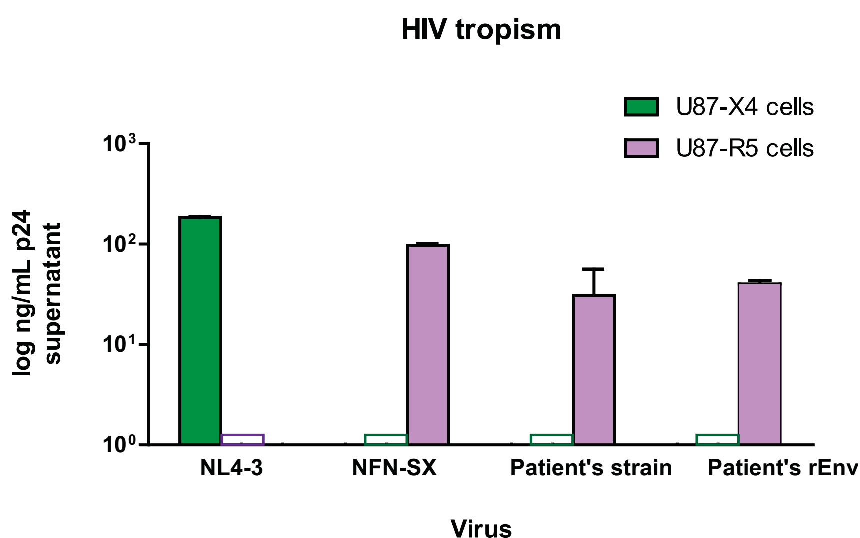


Figure 33. Determination of the viral tropisms from patients' HIV-1 isolates. Green bars show X4 tropism and purple bars show R5 tropism. White bars show the background of the detection technique.

3D.3.3. Latent reservoir

Sensitive assays were used to determine the latent reservoir of the patient pre and post-transplant (Table 9). Prior to transplantation, HIV-1 DNA could be detected in total and resting $CD4^+$ T-cells in peripheral blood as well as in gut-associated lymphoid tissue (GALT) biopsies. Additionally, we estimated that the replication competent viral size present in $CD4^+$ T-cells was 1.2 copies per ten million cells. We also detected cell-associated HIV-1 RNA in $CD4^+$ T-cells and HIV-1 RNA in plasma and cerebrospinal fluid. On day +76 after transplantation, with 100% *CCR5* Δ32/Δ32 CB chimerism, HIV-1 DNA was not detectable in isolated $CD4^+$ T-cells from peripheral blood using ddPCR quantification or semiquantitative test of amplification (Table 9). Unfortunately, DLBCL progression limited our capacity to extend our measurements of the viral reservoir to other tissues and long-term after HSCT.

Table 9. Latent HIV-1 reservoir measurements

	Day -15	Day +76
Total DNA (copies per million cells)		
Peripheral CD4$^+$ T cells	372.9	Undetectable
Peripheral resting CD4$^+$ T cells	651.8	ND
GALT CD4$^+$ T cells	803.2	ND
Semiquantitative total DNA (amplification %)		
Peripheral CD4$^+$ T cells	ND	0% (1:1, 1:10, 1:100, 1:1000)[a]
GALT	100% (1:1, 1:10, 1:100) 20% (1:1000)[a]	ND
Residual plasma viremia (copies per ml)		
Plasma	303	3.0
Cerebrospinal fluid	149	ND
Quantitative viral outgrowth (IUPM)		
Peripheral CD4$^+$ T cells	0.21[b]	ND
Peripheral resting CD4$^+$ T cells	<0.28[c]	ND
Cell-associated RNA (relative copies)		
Peripheral CD4$^+$ T cells	2.5	ND
GALT	Undetectable	ND

GALT: gut-associated lymphoid tissue
IUPM: infectious units per million cells
ND: non-determined for limited sample
[a] Proportion of positive PCRs of a total of 5 (1:10) or 10 (rest) replicas
[b] Cultured cells: 12 million
[c] Cultured cells: 2 million

3D.3.4. Cell recovery and Infectivity

Cord blood-derived patient's cells were evaluated after HSCT for activation/proliferation as well as infectivity (252). Thirty percent of the cells were able to proliferate and almost 50% expressed HLA-DR activation marker after PHA-stimulation (Figure 34A). However, activated CD4$^+$ T-cells were not infected by either the patient's autologous virus, or laboratory-adapted CCR5- or CXCR4-tropic viral strains (Figure 34B).

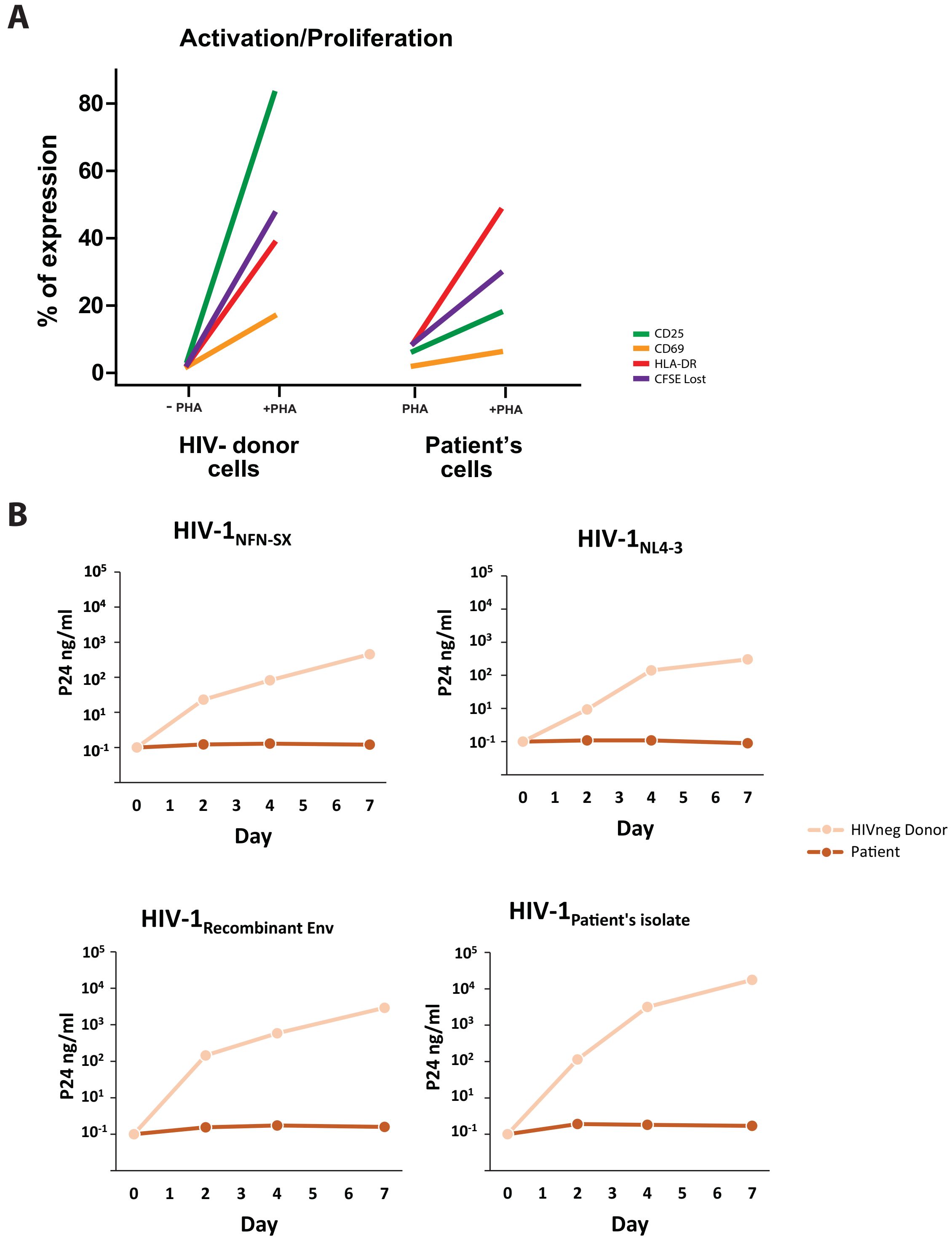


Figure 34. *CCR5* Genotype and Susceptibility to HIV-1 Infection. **(A)** Activation and proliferation of $CD4^+$ T-cells before and after PHA activation measured by flow cytometry. CD25, CD68 and HLA-DR markers are expressed as percent of $CD4^+$ T cells. Proliferation is measured as percent of cells with CFSE marker lost after activation. **(B)** Susceptibility of $CD4^+$ T cells to the infection with the patient's autologous virus isolate, the patient's Env-recombinant virus, and the laboratory-adapted strains HIV-1_{NFN-SX} (CCR5-tropic virus) and HIV-1_{NL4-3} (CXCR4-tropic virus).

3D.4. Discussion

A case of unrelated donor *CCR5* Δ32 homozygous allogeneic hematopoietic stem cell transplantation (HSCT) in 2009 raised new hopes for HIV-1 eradication (158,236). Five years later, a second case in 2014showed that control of HIV-1 by *CCR5* Δ32/Δ32 hematopoietic reconstitution may be compromised by viral co-receptor tropism and escape mechanisms (241,242). Adult HLA-compatible *CCR5* Δ32 homozygous donor availability will not suffice to investigate the conditions that drive functional cure of HIV-1 infection and to develop this therapeutic strategy further (160,161,243). Here, we describe a first reported case of allogeneic HSCT in an HIV-1-infected patient using *CCR5* Δ32/Δ32 hematopoietic stem cells from cord blood (CB), a stem cell source with more permissive HLA-matching requirements (244), which may provide a broader allogeneic HSCT platform for patients with HIV-1 infection. Up until now, two other attempted cases of *CCR5* Δ32 homozygous CB HSCT in HIV-infected patients, reported preliminary online and in abstract format (256,257), failed to show any impact on HIV-infection.

Allogeneic HSCT reduced our patient's latent viral reservoir, which became undetectable in circulating $CD4^+$ T cells both by semiquantitative amplification and ddPCR quantification tests (Table 9). Although myeloablative conditioning chemotherapy reduced viral levels prior to CB engraftment, evidence of HIV-1 recurrence upon discontinuation of ART in cases of allogeneic HSCT from *CCR5* heterozygous or wild-type donors questions the potential contribution from conditioning chemoradiotherapy, as well as from HSCT alloreactive events, to long-term control of HIV-1 infection (95,159,237,238). Despite the effect of HSCT on plasma viremia reduction, low HIV-1 RNA copy numbers were still detectable in the patient. Persistence of low-numbers of CCR5 wild-type cells below the detection level of chimerism analyses in tissues harboring infectious virus, long-lasting release of virus trapped in follicular dendritic cells in the lymph nodes, and the absence of longer-term follow-up are among the potential explanations for this finding.

In addition to undetectable HIV-1 DNA, upon achievement of full CB *CCR5* Δ32 homozygous chimerism, the patient's circulating $CD4^+$ T cells could be successfully stimulated to proliferate and express activation markers (Figure 34A), but were refractory to infection by HIV-1 (Figure 34B). The patient's $CD4^+$ T cells were re-

sistant to infection by the patient's HIV-1 isolates, by virus with the patient's recombinant Env, and by HIV-1_{NFN-SX} laboratory strain (all CCR5-tropic). They were also refractory to infection by the CXCR4-tropic laboratory strain HIV-1_{NL4-3}. Agrawal *et al.* (258) showed that $CD4^+$ T cells from *CCR5* Δ32 homozygous people consistently express low levels of CXCR4 and are less susceptible to infection by CXCR4-tropic HIV-1 than those individuals without the mutation (258). Petz *et al.* (246) also found that PBMCs from an HIV-1-negative patient with leukemia who had received a *CCR5* Δ32/Δ32 CB transplant were resistant *ex vivo* to HIV-1 infection with both CCR5-tropic and CXCR4-tropic HIV-1 strains (246). Beyond potential mechanisms of inhibition that could include scavenging of CXCR4 by endogenous expression of CCR5 Δ32 (258), the lack of infection by CXCR4 tropic viruses of otherwise functional reconstituted CD4+ T cells obtained shortly after achieving full chimerism is certainly intriguing and might be associated with maturation and functional stages of lymphocytes derived from hematopoietic stem cells from CB. These points towards potential unknown additional mechanisms require further investigation.

Undoubtedly, standards of care for allogeneic HSCT in the primary indication for allogeneic HSCT must be the priority, even when considering patients with HIV-1 infection. However, although HIV-infected patients remain at an increased risk of hematologic malignancies for which HSCT is considered standard therapy, the outcome of allogeneic HSCT in patients with HIV-1 infection is largely unknown. The European Society for Bone Marrow Transplantation (EBMT) has very recently communicated a large pair-matched cohort study with 111 allogeneic HSCT recipients with HIV-1 infection each matched with three controls, which showed poor overall outcomes for patients with HIV-1 infection (259). These results seem to be driven by higher non-relapse mortality and higher rates of severe GVHD than in uninfected transplant recipients. Nevertheless, with a 47% overall survival at 2 years, allogeneic HSCT is feasible in HIV-infected individuals, and the outcome is comparable between related and unrelated donor sources. Cord blood transplantation has become a standard source of alternative grafts for allogeneic HSCT in patients without matched sibling donors (260). In combination with haploidentical hematopoietic cells, CB reliably leads to faster neutrophil engraftment than does alternative single or double-CB transplant protocols, and has similar

overall outcomes while allowing the use of CB units with somewhat lower cellular content (244,253–255). This approach provides a major advantage in the setting of HIV-1 infection, in which projections suggest that an inventory of 300 *CCR5* Δ32 homozygous cord blood units would allow, with this protocol, an adequate HLA-matched unit for over 80% of HIV-1-infected adult and pediatric transplant candidates (246). The case we report shows that a directed CB search and *CCR5* Δ32 homozygous CB transplantation is viable in patients with HIV-1 infection, can make viral DNA undetectable and can render the patient's CB-derived $CD4^+$ T lymphocytes refractory to infection by HIV-1. Despite lymphoma progression making long-term assessment of HIV-1 infection unfeasible, in combination with the evidence available, our findings support the feasibility of CB transplantation as a therapeutic strategy to cure HIV-1 infection, and as a platform to investigate key patient-derived, HIV-dependent, and treatment-associated factors that might drive a broader application of *CCR5*-knockout strategies to patients with HIV-1.

CHAPTER 4. GLOBAL DISCUSSION AND PERSPECTIVES

Combination antiretroviral therapy (ART) was one of the major medical successes of the 20th century, as it efficiently suppresses HIV-1 replication, restores the immune system, and reduces drug resistance, clinical failure, viral rebound, mortality and morbidity, and viral transmission (45,47–52). However, despite a life-long treatment regimen, ART cannot eradicate the HIV-1 reservoir and, thus, in most individuals, viral load rapidly rebounds after treatment interruption. Therefore, the development of novel approaches that are able to cure this infection is the major objective of the HIV-1 research field.

Only one approach has achieved the sterilizing cure of an HIV-1 infected patient, the “Berlin patient”. Nonetheless, the performance of a hematopoietic stem cell transplantation (HSCT) and the compromised medical situation of patients, who suffer HIV-1 infection and leukemia or lymphoma, make it quite difficult to replicate this case. Moreover, this cure strategy was a probe of concept as HSCT is not feasible and accessible for all HIV-1 infected patients. Hence, new strategies, focused on the seeking of a functional or a sterilizing cure, are being developed. These approaches include treatment optimization - by intensification, switching or early initiation of ART -, immunotherapy, latency reactivation, therapeutic vaccination, and/or cellular or gene therapy.

This thesis proposed to evaluate four treatment strategies based on immunotherapy, latency reactivation, therapeutic vaccination or cellular therapy, which may have an impact on the HIV-1 reservoir, and, thus, may be used as a promising HIV-1 cure strategy.

As immunotherapy, we analyzed the effect of short-term treatment with the cytokine interferon-α (IFN-α) in co−infected HIV/HCV chronically ART-suppressed subjects, and the mechanism by which long-term treatment with IFN-α decreases the total HIV-1 reservoir size (180,181). Our results showed that short-term administration of IFN-α decreases HIV-1 transcription and T-cell activation, although it does not relevantly impact the HIV-1 reservoir. Thus, IFN-α may act using different mechanisms over time, with short-term therapy acting more as a viral repressor than as a reactivating agent. Hence, IFN-α treatment may be used as immunotherapy combined with ART to increase viral repression and aid to improve the

immune system's state, and it may even potentially achieve a functional cure after long-term treatment.

Regarding latency reactivation, we analyzed the impact of lithium treatment on the HIV-1 reservoir in chronically ART suppressed patients. Previous studies have related lithium with inhibiting viral replication and reactivating viral latency *in vitro* (191–193). Thus, we aimed to evaluate whether lithium carbonate may act as a latency-reversing agent (LRA) when administered *in vivo*. Our results showed that *in vivo* lithium can transiently reduce HIV-1 transcription and total HIV-1 reservoir, but has no long-term effect on the HIV-1 reservoir size. Therefore, *in vivo* lithium treatment does not reactivate the HIV-1 latent proviruses, but it acts as a transcriptional repressor of active proviruses, which constitute residual viral replication. Hence, lithium might be proposed as an intensification of ART in intermittent doses given that it affects residual HIV-1 replication likely due to its better tissue penetration among other mechanisms.

Considering therapeutic vaccination, we evaluated the effects of immunization with the conserved immunogen HIVconsv vectored by the modified vaccinia virus Ankara vector (MVA.HIVconsv) in chronically ART suppressed patients. This was the first study that analyzed the immunization with the HIVconsv immunogen in HIV-1 infected patients, and showed that MVA.HIVconsv can elicit specific HIV-1 immune responses against conserved non-common epitopes. However, these immune responses were not able to affect the total HIV-1 reservoir or the residual plasma viremia, due to its inefficacy against the latently infected cells. Therefore, this therapeutic vaccine would have to be combined with LRAs administration to be able to mediate the immune clearance of those latently infected cells, thus achieving a remission of HIV-1 infection.

Regarding cellular therapy, we evaluated the case report of an ART suppressed patient suffering from a diffuse large B-cell lymphoma (DLBCL), who received an HSCT with *CCR5* homozygous Δ32 cord blood. Our results showed that after transplantation total HIV-1 reservoir was undetectable, and the patient's circulating $CD4^+$ T cells were refractory to infection by HIV-1 *ex vivo*, suggesting that the patient may have been cured of the HIV-1 infection. However, we were not able to perform further analysis given that the patient passed away due to pro-

gression of his hematological disease three months after transplant. Therefore, the use of HIV-1 resistant cord blood units for HSCT may achieve a functional or sterilizing cure, as was the case with the "Berlin patient", despite all the difficulties involved in finding HLA-matched HIV-1 resistant cord blood units, and the compromised immune situation of the subjects.

Overall, after the evaluation of these four clinical trials, we can summarize that immunotherapy with IFN-α and treatment with lithium may be used in combination with ART as treatment intensification, but they are not able to achieve a functional cure on their own. Moreover, therapeutic vaccination with MVA.HIVconsv immunization can elicit new immune responses that might mediate an immune clearance of the latently infected cells, but it should be administered in combination with a LRA to achieve an efficient "shock and kill" of the reservoir and thus a functional or sterilizing cure. Finally, HSCT with *CCR5* homozygous Δ32 cord blood may be the approach with the highest probability of achieving a sterilizing HIV-1 cure, given that after HSCT engraftment we could not detect cell-associated HIV-1 DNA in the patient's $CD4^+$ T cells.

In this context, some future perspectives among HIV-1 cure strategies remain open. Firstly and foremost, it is necessary to develop LRAs that effectively reactivate all the replication-competent proviruses and that have an effect both in the blood and in the lymphoid tissues, where most of the latently infected cells reside (261). Secondly, a therapeutically active vaccine, able to elicit potent and broad T and B cell immune responses, needs to be developed (140). And, thirdly, a combination of broadly neutralizing antibodies has to be designed to be administered as therapeutic passive immunization in combination with therapeutic active vaccine and LRAs (262). Moreover, all these improved strategies should be combined with immunotherapy, such as anti-PD-1 or anti-PD-L1 antibodies, to improve and restore the immune status of chronically ART-treated patients (137). Alternatively, the should be combined with early treatment initiation, to keep the HIV-1 reservoir as smallest as possible (118). The smaller the reservoir is, the easier it will be to eliminate all the replication-competent proviruses and, thus, to achieve a functional or a sterilizing cure.

Furthermore, it is necessary to test the cord blood units (CBU) stored at BioBank facilities to register which ones have the 32 bp deletion in *CCR5*, and thus facilitate the finding of an HIV-1 resistant CBU HLA-matched with a subject suffering HIV-1 infection and lymphoma or leukemia (162). However, this approach can only be performed in HIV-1 infected individuals who have been diagnosed with a hematologic malignancy and have already received at least one autologous HSCT. Therefore, HIV-1 researchers also need to develop the best possible gene therapy strategy to modify autologous hematopoietic cells of HIV-1 infected individuals, to restore their immune system with HIV-1 resistant cells or to purge the HIV-1 provirus from the latently infected cells (263). This last strategy may be applicable to all the HIV-1 infected individuals, regardless of them suffering from a hematopoietic disease or not.

In summary, the optimal scenario for achieving a functional or a sterilizing cure would be to begin treatment with ART as promptly as possible, thereby quickly suppressing HIV-1 viral load and limiting the establishment of the viral reservoir and the levels of immune activation in the early stages of the infection (118), allowing for a good restoration of the immune system (high $CD4^+$ T-cell counts and a ratio CD4/CD8 between 1 and 2). However, the functional or the sterilizing cure would be also possible in chronically ART-treated patients, with or without a low viral reservoir, by combining different strategies to restore the immune system, decreasing the persistent immune activation associated with the chronic phases of HIV-1 infection, eliciting broad HIV-1 specific immune responses, and reactivating the latently-infected cells. Thus, the HIV-1 cure is not centered on a unique therapeutic approach, but on a combination of multiple clinical strategies that can impact the HIV-1 reservoir and enhance specific immune responses against this infection.

CHAPTER 5. CONCLUSIONS

With the aim to evaluate and determining which HIV-1 cure strategy may achieve a sterilizing or a functional cure, we compared four clinical trials based on immunotherapy, latency reactivation, therapeutic vaccination and cellular therapy.

The **conclusions** for each cure strategy analyzed are:

1. Immunotherapy:

Conclusion of aim 1.1. Short-term treatment with interferon-α combined with ribavirin can trigger a reduction in HIV-1 transcription in HIV/HCV–coinfected patients on suppressive combination ART through up-regulation of viral restriction genes, mainly *TRIM22*, and a decrease in T-cell activation.

Conclusion of aim 1.2. After 28 days of treatment, interferon-α acts more as a viral suppressor than as a reactivating agent.

2. Latency reactivation:

Conclusion of aim 2.1. Potential treatment of HIV-1-associated neurocognitive abnormalities with lithium carbonate transiently represses HIV-1 transcription and decreases the total HIV-1 reservoir, in ART-suppressed patients, possibly as a result of a cellular adaptive response to the chronic activation of the β-catenin signaling pathway.

Conclusion of aim 2.2. Unexpectedly, lithium carbonate treatment *in vivo* transiently acts as an HIV-1 transcription repressor rather than as a latency-reversing agent (LRA).

3. Therapeutic vaccination:

Conclusion of aim 3.1. Therapeutic vaccination with MVA.HIVconsv could redirect T-cell responses towards viral epitopes that are sub-dominant in natural infection, given that interferon-γ T cell responses to conserved epitopes were increased after vaccination, in ART suppressed HIV-infected patients.

Conclusion of aim 3.2. However, the sub-dominant specific HIV-1 immune responses elicited have no impact on the total HIV-1 reservoir or the residual plasma viremia.

4. Cellular therapy:

Conclusion of aim 4.1. Allogeneic hematopoietic stem cell transplantation (HSCT) using *CCR5* Δ32 homozygous cord blood (CB) cells can be a feasible therapeutic strategy to cure HIV-1 infection, and a platform to investigate the key patient-derived factors that may drive a broader application of *CCR5* knockdown gene therapy strategies to patients with HIV-1 infection.

Conclusion of aim 4.2. Allogeneic HSCT using a compatible *CCR5* Δ32 homozygous CB reduced the patient's latent total viral reservoir in peripheral $CD4^+$ T cells to undetectable levels. However, the inability to test later samples, due to the fatal outcome of the patient from aggressive progression of the diffuse large B-cell lymphoma, prevented us from concluding that allogeneic HSCT using a compatible *CCR5* Δ32 homozygous CB could effectively eradicate the HIV-1 infection.

Globally, our results showed that the strategy with the highest probability of achieving a sterilizing or a functional cure would appear to be the allogeneic hematopoietic stem cell transplantation using *CCR5* Δ32 homozygous bone marrow or cord blood cells. Until to date, immunotherapy, latency reactivation and therapeutic vaccination strategies evaluated, on their own, have not shown sufficient potency to reduce the size of the reservoir. Thus, none of these approaches have achieved a functional or a sterilizing cure yet. Hence, the combination of these strategies may be the next step to finally achieve a remission of the HIV-1 infection.

CHAPTER 6. REFERENCES

1. Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA, et al. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N Engl J Med. 1981 Dec 10;305(24):1425–31.

2. Centers for Disease Control. Pneumocystis Pneumonia - Los Angeles. Morb Mortal Wkly Rep. 1981;30(6):250–2.

3. Barré-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, et al. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science. 1983 May 20;220(4599):868–71.

4. Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, et al. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science. 1984 May 4;224(4648):500–3.

5. Levy JA, Hoffman AD, Kramer SM, Landis JA, Shimabukuro JM, Oshiro LS. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science. 1984 Aug 24;225(4664):840–2.

6. Coffin J, Haase A, Levy JA, Montagnier L, Oroszlan S, Teich N, et al. Human immunodeficiency viruses. Science. 1986 May 9;232(4751):697.

7. Korber B, Muldoon M, Theiler J, Gao F, Gupta R, Lapedes A, et al. Timing the ancestor of the HIV-1 pandemic strains. Science. 2000 Jun 9;288(5472):1789–96.

8. Lemey P, Pybus OG, Wang B, Saksena NK, Salemi M, Vandamme A-M. Tracing the origin and history of the HIV-2 epidemic. Proc Natl Acad Sci. 2003 May 12;100(11):6588–92.

9. Faria NR, Rambaut A, Suchard MA, Baele G, Bedford T, Ward MJ, et al. The early spread and epidemic ignition of HIV-1 in human populations. Science (80-). 2014 Oct 2;346(6205):56–61.

10. Hahn BH, Shaw GM, De Cock KM, Sharp PM. AIDS as a zoonosis: scientific and public health implications. Science. 2000 Jan 28;287(5453):607–14.

11. Clavel F, Guétard D, Brun-Vézinet F, Chamaret S, Rey MA, Santos-Ferreira MO, et al. Isolation of a new human retrovirus from West African patients with AIDS. Science. 1986 Jul 18;233(4761):343–6.

12. Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, Michael SF, et al. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature. 1999 Feb 4;397(6718):436–41.

13. Gao F, Yue L, Robertson DL, Hill SC, Hui H, Biggar RJ, et al. Genetic diversity of human immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. J Virol. 1994 Nov;68(11):7433–47.

14. International Committee on Taxonomy of Viruses. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. King A, Adams M, Carstens E, Lefkowitz E, editors. San Diego: Elsevier Academic Press; 2012.

15. Reeves JD, Doms RW. Human immunodeficiency virus type 2. J Gen Virol. 2002 Jun;83(Pt 6):1253–65.

16. Robertson DL, Anderson JP, Bradac JA, Carr JK, Foley B, Funkhouser RK, et al. HIV-1 nomenclature proposal. Science. 2000 Apr 7;288(5463):55–6.

17. Worobey M, Telfer P, Souquière S, Hunter M, Coleman CA, Metzger MJ, et al. Island biogeography reveals the deep history of SIV. Science. 2010 Sep 17;329(5998):1487.

18. UNAIDS. Fact Sheet Report 2015. 2015.

19. Muesing MA, Smith DH, Cabradilla CD, Benton C V, Lasky LA, Capon DJ. Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. Nature. Jan;313(6002):450–8.

20. Splettstoesser T. SciStyle Scientific Illustration [Internet]. Available from: http://www.scistyle.com

21. Abfalterer W, Athreya G, Fischer W, Funkhouser B, Lo C-C, Macke J, et al. HIV sequence compendium 2009. Kuiken C, Foley B, Marx P, Wolinsky S, Leitner T, Hahn BH, et al., editors. Los Alamos: Theoretical Biology and Biophysics, Los Alamos National Laboratory; 2010. 432 p.

22. Altfeld M, Barmeyer A, Behrens G, Berzow D, Boesecke C, Braun P, et al. HIV 2015/16. Hoffmann C, Rockstroh JK, Camp R, editors. Hamburg: Medizin Fokus Verlag; 2015. 776 p.

23. Arthur LO, Bess JW, Sowder RC, Benveniste RE, Mann DL, Chermann JC, et al. Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. Science. 1992 Dec 18;258(5090):1935–8.

24. Turner BG, Summers MF. Structural biology of HIV. J Mol Biol. 1999 Jan 8;285(1):1–32.

25. Maddon PJ, Dalgleish AG, McDougal JS, Clapham PR, Weiss RA, Axel R. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell. 1986 Nov 7;47(3):333–48.

26. Chan DC, Kim PS. HIV entry and its inhibition. Cell. 1998 May 29;93(5):681–4.

27. Hu W-S, Hughes SH. HIV-1 reverse transcription. Cold Spring Harb Perspect Med. 2012 Oct;2(10).

28. Craigie R, Bushman FD. HIV DNA integration. Cold Spring Harb Perspect Med. 2012 Jul;2(7):a006890.

29. Varmus H. Retroviruses. Science. 1988 Jun 10;240(4858):1427–35.

30. Leblanc J, Weil J, Beemon K. Posttranscriptional regulation of retroviral gene expression: primary RNA transcripts play three roles as pre-mRNA, mRNA, and genomic RNA. Wiley Interdiscip Rev RNA. 2013 Jan;4(5):567–80.

31. Karn J, Stoltzfus CM. Transcriptional and posttranscriptional regulation of HIV-1 gene expression. Cold Spring Harb Perspect Med. 2012 Feb;2(2):a006916.

32. Sundquist WI, Kräusslich H-G. HIV-1 assembly, budding, and maturation. Cold Spring Harb Perspect Med. 2012 Jul;2(7):a006924.

33. Shen R, Richter HE, Smith PD. Early HIV-1 target cells in human vaginal and ectocervical mucosa. Am J Reprod Immunol. 2011 Mar;65(3):261–7.

34. Joseph SB, Arrildt KT, Sturdevant CB, Swanstrom R. HIV-1 target cells in the CNS. J Neurovirol. 2015 Jun;21(3):276–89.

35. Streeck H, Altfeld M. Acute HIV-1 Infection. In: Hoffmann C, Rockstroh JK, Camp R, editors. HIV 2015/16. Hamburg: Medizin Fokus Verlag; 2015. p. 52–61.

36. McMichael AJ, Borrow P, Tomaras GD, Goonetilleke N, Haynes BF. The immune

response during acute HIV-1 infection: clues for vaccine development. Nat Rev Immunol. 2010 Jan;10(1):11–23.

37. Babiker A, Darby S, De Angelis D, Kwart D, Porter K, Beral V, et al. Time from HIV-1 seroconversion to AIDS and death before widespread use of highly-active antiretroviral therapy: a collaborative re-analysis. Collaborative Group on AIDS Incubation and HIV Survival including the CASCADE EU Concerted Action. Concerted Actio. Lancet. 2000 Apr 1;355(9210):1131–7.

38. Bajaria SH, Webb G, Cloyd M, Kirschner D. Dynamics of naive and memory CD4+ T lymphocytes in HIV-1 disease progression. J Acquir Immune Defic Syndr. 2002 May 1;30(1):41–58.

39. Relucio K, Holodniy M. HIV-1 RNA and viral load. Clin Lab Med. 2002 Sep;22(3):593–610.

40. Fauci A, Lane H. Chapter 173. Human Immunodeficiency Virus Disease: AIDS and Related Disorders. In: Kasper DL, editor. Harrison's Principles of Internal Medicine. 16th Vol.1 ed. McGraw-Hill; 2005. p. 1076–139.

41. Langford SE, Ananworanich J, Cooper DA. Predictors of disease progression in HIV infection: a review. AIDS Res Ther. 2007 Jan;4:11.

42. Baltimore D, Boeke JD, Brown PO, Desrosiers R, Emini EA, Fan HY, et al. Course of Infection with HIV and SIV. In: Coffin JM, Hughes SH, Varmus HE, editors. Retroviruses. Cold Spring Harbor Laboratory Press; 1997.

43. Hoffmann C. AIDS. In: Hoffmann C, Rockstroh JK, Camp R, editors. HIV 2015/16. Hamburg: Medizin Fokus Verlag; 2015. p. 331–449.

44. Molotsky I. U.S. approves drug to prolong lives of AIDS patients. New York Times. Washington; 1987 Mar 21;

45. Arts EJ, Hazuda DJ. HIV-1 Antiretroviral Drug Therapy. Cold Spring Harb Perspect Med. 2012 Jan 25;2(4):a007161–a007161.

46. U.S. Food and Drug Administration, U.S. Department of Health and Human Services. HIV/AIDS Treatment - Antiretroviral drugs used in the treatment of HIV infection [Internet]. Silver Spring; 2016 [cited 2016 May 23]. Available from: http://www.fda.gov/ForPatients/Illness/HIVAIDS/Treatment/ucm118915.htm

47. Autran B, Carcelain G, Li TS, Blanc C, Mathez D, Tubiana R, et al. Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease. Science. 1997 Jul 4;277(5322):112–6.

48. Komanduri K V, Viswanathan MN, Wieder ED, Schmidt DK, Bredt BM, Jacobson MA, et al. Restoration of cytomegalovirus-specific CD4+ T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1. Nat Med. 1998 Aug;4(8):953–6.

49. Lederman MM, Connick E, Landay A, Kuritzkes DR, Spritzler J, St Clair M, et al. Immunologic responses associated with 12 weeks of combination antiretroviral therapy consisting of zidovudine, lamivudine, and ritonavir: results of AIDS Clinical Trials Group Protocol 315. J Infect Dis. 1998 Jul;178(1):70–9.

50. Johnson VA. Combination therapy: more effective control of HIV type 1? AIDS Res Hum Retroviruses. 1994 Aug;10(8):907–12.

51. Palella FJ, Armon C, Buchacz K, Chmiel JS, Novak RM, D'Aquila RT, et al. Factors associated with mortality among persistently viraemic triple-antiretroviral-class-experienced patients receiving antiretroviral therapy in the HIV Outpatient Study (HOPS). J Antimicrob Chemother. 2014 Oct;69(10):2826–34.

52. Williams BG, Lima V, Gouws E. Modelling the impact of antiretroviral therapy on the epidemic of HIV. Curr HIV Res. 2011 Sep;9(6):367–82.

53. Martin AR, Siliciano RF. Progress Toward HIV Eradication: Case Reports, Current Efforts, and the Challenges Associated with Cure. Annu Rev Med. 2016 Nov 2;(67):215–28.

54. Martinez-Picado J, Deeks SG. Persistent HIV-1 replication during antiretroviral therapy. Curr Opin HIV AIDS. 2016;

55. Fletcher C V, Staskus K, Wietgrefe SW, Rothenberger M, Reilly C, Chipman JG, et al. Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues. Proc Natl Acad Sci U S A. 2014 Mar 11;111(6):2307–12.

56. Deeks SG, Autran B, Berkhout B, Benkirane M, Cairns S, Chomont N, et al. Towards an HIV cure: a global scientific strategy. Nat Rev Immunol. 2012;12(8):607–14.

57. Chun TW, Finzi D, Margolick J, Chadwick K, Schwartz D, Siliciano RF. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. Nat Med. 1995 Dec;1(12):1284–90.

58. Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, et al. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science. 1997 Nov 14;278(5341):1295–300.

59. Restifo NP, Gattinoni L. Lineage relationship of effector and memory T cells. Curr Opin Immunol. 2013 Oct;25(5):556–63.

60. Ahmed R, Bevan MJ, Reiner SL, Fearon DT. The precursors of memory: models and controversies. Nat Rev Immunol. 2009 Sep;9(9):662–8.

61. Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, et al. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nat Med. 2009 Aug;15(8):893–900.

62. Kumar A, Abbas W, Herbein G. HIV-1 Latency in Monocytes/Macrophages. Viruses. 2014 Jan;6(4):1837–60.

63. Miles B, Connick E. TFH in HIV Latency and as Sources of Replication-Competent Virus. Trends Microbiol. 2016 May;24(5):338–44.

64. Barton K, Winckelmann A, Palmer S. HIV-1 Reservoirs During Suppressive Therapy. Trends Microbiol. 2016 Feb 11;24(5):345–55.

65. Strain MC, Little SJ, Daar ES, Havlir D V, Gunthard HF, Lam RY, et al. Effect of treatment, during primary infection, on establishment and clearance of cellular reservoirs of HIV-1. J Infect Dis. 2005 May 1;191(9):1410–8.

66. Massanella M, Richman DD. Measuring the latent reservoir in vivo. J Clin Invest. 2016;126(2).

67. Bruner KM, Hosmane NN, Siliciano RF. Towards an HIV-1 cure: measuring the latent reservoir. Trends Microbiol. Elsevier Ltd; 2015 Mar;1–12.

68. Eriksson S, Graf EH, Dahl V, Strain MC, Yukl SA, Lysenko ES, et al. Comparative analysis of measures of viral reservoirs in HIV-1 eradication studies. PLoS Pathog. 2013 Feb;9(2):e1003174.

69. Rouzioux C, Mélard A, Avéttand-Fénoël V. Quantification of total HIV1-DNA in peripheral blood mononuclear cells. Methods Mol Biol. 2014 Jan;1087:261–70.

70. Strain MC, Lada SM, Luong T, Rought SE, Gianella S, Terry VH, et al. Highly precise measurement of HIV DNA by droplet digital PCR. PLoS One. 2013 Jan;8(4):e55943.

71. Ho Y-C, Laird GM, Siliciano RF. Measuring reversal of HIV-1 latency ex vivo using cells from infected individuals. Proc Natl Acad Sci U S A. 2014 May 13;111(19):6860–1.

72. O'Doherty U, Swiggard WJ, Jeyakumar D, McGain D, Malim MH. A sensitive, quantitative assay for human immunodeficiency virus type 1 integration. J Virol. 2002 Nov;76(21):10942–50.

73. Liszewski MK, Yu JJ, O'Doherty U. Detecting HIV-1 integration by repetitive-sampling Alu-gag PCR. Methods. 2009;47(4):254–60.

74. Brady T, Kelly BJ, Male F, Roth S, Bailey A, Malani N, et al. Quantitation of HIV DNA integration: effects of differential integration site distributions on Alu-PCR assays. J Virol Methods. 2013 Apr;189(1):53–7.

75. Koelsch KK, Liu L, Haubrich R, May S, Havlir D, Günthard HF, et al. Dynamics of total, linear nonintegrated, and integrated HIV-1 DNA in vivo and in vitro. J Infect Dis. 2008 Feb 1;197(3):411–9.

76. Vandegraaff N, Kumar R, Burrell CJ, Li P. Kinetics of human immunodeficiency virus type 1 (HIV) DNA integration in acutely infected cells as determined using a novel assay for detection of integrated HIV DNA. J Virol. 2001 Nov;75(22):11253–60.

77. Chun T-W, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, et al. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature. 1997 May 8;387(6629):183–8.

78. Buzón MJ, Massanella M, Llibre JM, Esteve A, Dahl V, Puertas MC, et al. HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects. Nat Med. 2010;16(4):460–5.

79. Hatano H, Strain MC, Scherzer R, Bacchetti P, Wentworth D, Hoh R, et al. Increase

in 2-long terminal repeat circles and decrease in D-dimer after raltegravir intensification in patients with treated HIV infection: a randomized, placebo-controlled trial. J Infect Dis. 2013 Nov 1;208(9):1436–42.

80. Soriano-Sarabia N, Archin NM, Bateson R, Dahl NP, Crooks AM, Kuruc JD, et al. Peripheral Vγ9Vδ2 T Cells Are a Novel Reservoir of Latent HIV Infection. PLoS Pathog. 2015 Oct;11(10):e1005201.

81. Badralmaa Y, Natarajan V. Impact of the DNA extraction method on 2-LTR DNA circle recovery from HIV-1 infected cells. J Virol Methods. Elsevier B.V.; 2013;193(1):184–9.

82. Fischer M, Joos B, Wong JK, Ott P, Opravil M, Hirschel B, et al. Attenuated and nonproductive viral transcription in the lymphatic tissue of HIV-1-infected patients receiving potent antiretroviral therapy. J Infect Dis. 2004 Jan 15;189(2):273–85.

83. Pasternak AO, Adema KW, Bakker M, Jurriaans S, Berkhout B, Cornelissen M, et al. Highly sensitive methods based on seminested real-time reverse transcription-PCR for quantitation of human immunodeficiency virus type 1 unspliced and multiply spliced RNA and proviral DNA. J Clin Microbiol. 2008 Jul;46(7):2206–11.

84. Pasternak AO, Jurriaans S, Bakker M, Prins JM, Berkhout B, Lukashov V V. Cellular levels of HIV unspliced RNA from patients on combination antiretroviral therapy with undetectable plasma viremia predict the therapy outcome. PLoS One. 2009 Jan;4(12):e8490.

85. Schmid A, Gianella S, von Wyl V, Metzner KJ, Scherrer AU, Niederöst B, et al. Profound depletion of HIV-1 transcription in patients initiating antiretroviral therapy during acute infection. PLoS One. 2010;5(10).

86. Gianella S, Massanella M, Richman DD, Little SJ, Spina CA, Vargas M V, et al. Cytomegalovirus replication in semen is associated with higher levels of proviral HIV DNA and CD4+ T cell activation during antiretroviral treatment. J Virol. 2014 Jul;88(14):7818–27.

87. Puertas, MC.; Salgado, M; Morón-López, S; Ouchi, D; Muñoz-Moreno, J; Moltó, J; Clotet, B; Martinez-Picado J. Effect of lithium on HIV-1 expression and proviral reservoir size in the CD4 + T cells of ART-suppressed patients. AIDS. 2014;

88. Gianella S, Anderson CM, Vargas M V, Richman DD, Little SJ, Morris SR, et al.

Cytomegalovirus DNA in semen and blood is associated with higher levels of proviral HIV DNA. J Infect Dis. 2013 Mar 15;207(6):898–902.

89. Bullen CK, Laird GM, Durand CM, Siliciano JD, Siliciano RF. New ex vivo approaches distinguish effective and ineffective single agents for reversing HIV-1 latency in vivo. Nat Med. Nature Publishing Group; 2014 Apr;20(4):425–9.

90. Shan L, Rabi SA, Laird GM, Eisele EE, Zhang H, Margolick JB, et al. A novel PCR assay for quantification of HIV-1 RNA. J Virol. 2013 Jun;87(11):6521–5.

91. Pasternak AO, Lukashov V V, Berkhout B. Cell-associated HIV RNA: a dynamic biomarker of viral persistence. Retrovirology. 2013 Jan;10:41.

92. Sahu GK, Sarria JC, Cloyd MW. Recovery of replication-competent residual HIV-1 from plasma of a patient receiving prolonged, suppressive highly active antiretroviral therapy. J Virol. 2010 Aug;84(16):8348–52.

93. Palmer S, Wiegand AP, Maldarelli F, Bazmi H, Mican JM, Polis M, et al. New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. J Clin Microbiol. 2003 Oct;41(10):4531–6.

94. Palmer S. Advances in detection and monitoring of plasma viremia in HIV-infected individuals receiving antiretroviral therapy. Curr Opin HIV AIDS. 2013 Mar;8(2):87–92.

95. Cillo AR, Krishnan S, McMahon DK, Mitsuyasu RT, Para MF, Mellors JW. Impact of chemotherapy for HIV-1 related lymphoma on residual viremia and cellular HIV-1 DNA in patients on suppressive antiretroviral therapy. PLoS One. 2014 Jan;9(3):e92118.

96. Amendola A, Bloisi M, Marsella P, Sabatini R, Bibbò A, Angeletti C, et al. Standardization and performance evaluation of "modified" and "ultrasensitive" versions of the Abbott RealTime HIV-1 assay, adapted to quantify minimal residual viremia. J Clin Virol. Elsevier B.V.; 2011 Sep;52(1):17–22.

97. Yukl SA, Li P, Fujimoto K, Lampiris H, Lu CM, Hare CB, et al. Modification of the Abbott RealTime assay for detection of HIV-1 plasma RNA viral loads less than one copy per milliliter. J Virol Methods. Elsevier B.V.; 2011 Aug;175(2):261–5.

98. Paba P, Fabeni L, Ciccozzi M, Perno CF, Ciotti M. Performance evaluation of the COBAS/TaqMan HIV-1 v2.0 in HIV-1 positive patients with low viral load: a comparative study. J Virol Methods. Elsevier B.V.; 2011 May;173(2):399–402.

99. Karasi JC, Dziezuk F, Quennery L, Förster S, Reischl U, Colucci G, et al. High correlation between the Roche COBAS® AmpliPrep/COBAS® TaqMan® HIV-1, v2.0 and the Abbott m2000 RealTime HIV-1 assays for quantification of viral load in HIV-1 B and non-B subtypes. J Clin Virol. 2011 Nov;52(3):181–6.

100. Pyne MT, Wilson A, Hillyard DR. Large-scale comparison of Roche Cobas AmpliPrep/Cobas TaqMan and Abbott RealTime HIV assays. J Virol Methods. Elsevier B.V.; 2012 Sep;184(1-2):106–8.

101. Nugent CT, Dockter J, Bernardin F, Hecht R, Smith D, Delwart E, et al. Detection of HIV-1 in alternative specimen types using the APTIMA HIV-1 RNA Qualitative Assay. J Virol Methods. 2009 Jul;159(1):10–4.

102. Siliciano JD, Siliciano RF. Enhanced culture assay for detection and quantitation of latently infected, resting CD4+ T-cells carrying replication-competent virus in HIV-1-infected individuals. Methods Mol Biol. 2005 Jan;304:3–15.

103. Laird GM, Eisele EE, Rabi SA, Lai J, Chioma S, Blankson JN, et al. Rapid quantification of the latent reservoir for HIV-1 using a viral outgrowth assay. PLoS Pathog. 2013 Jan;9(5):e1003398.

104. Prado JG, Prendergast A, Thobakgale C, Molina C, Tudor-Williams G, Ndung'u T, et al. Replicative capacity of human immunodeficiency virus type 1 transmitted from mother to child is associated with pediatric disease progression rate. J Virol. 2010 Jan;84(1):492–502.

105. Dalmau J, Codoñer FM, Erkizia I, Pino M, Pou C, Paredes R, et al. In-depth characterization of viral isolates from plasma and cells compared with plasma circulating quasispecies in early HIV-1 infection. PLoS One. 2012 Jan;7(2):e32714.

106. Metcalf Pate KA, Pohlmeyer CW, Walker-Sperling VE, Foote JB, Najarro KM, Cryer CG, et al. A Murine Viral Outgrowth Assay to Detect Residual HIV Type 1 in Patients With Undetectable Viral Loads. J Infect Dis. 2015 Nov 1;212(9):1387–96.

107. Procopio FA, Fromentin R, Kulpa D a, Brehm JH, Bebin A-G, Strain MC, et al. A Novel Assay to Measure the Magnitude of the Inducible Viral Reservoir in HIV-

infected Individuals. EBioMedicine. 2015 Aug;2(8):872–81.

108. Grossman CI, Ross AL, Auerbach JD, Ananworanich J, Dubé K, Tucker JD, et al. Towards Multidisciplinary HIV-Cure Research: Integrating Social Science with Biomedical Research. Trends Microbiol. 2016 Jan;24(1):5–11.

109. Puertas MC, Massanella M, Llibre JM, Ballestero M, Buzon MJ, Ouchi D, et al. Intensification of a raltegravir-based regimen with maraviroc in early HIV-1 infection. AIDS. 2014 Jan 28;28(3):325–34.

110. Markowitz M, Evering TH, Garmon D, Caskey M, La Mar M, Rodriguez K, et al. A randomized open-label study of 3- versus 5-drug combination antiretroviral therapy in newly HIV-1-infected individuals. J Acquir Immune Defic Syndr. 2014 Jun 1;66(2):140–7.

111. Phanuphak N, Teeratakulpisarn N, van Griensven F, Chomchey N, Pinyakorn S, Fletcher JL, et al. Anogenital HIV RNA in Thai men who have sex with men in Bangkok during acute HIV infection and after randomization to standard vs. intensified antiretroviral regimens. J Int AIDS Soc. 2015 Jan;18(1):19470.

112. Lam YMP, McBride KL, Amin J, Cordery D V, Kelleher AD, Cooper DA, et al. Switching virally suppressed, treatment-experienced patients to a raltegravir-containing regimen does not alter levels of HIV-1 DNA. PLoS One. 2012 Jan;7(3):e31990.

113. Bianco C, Meini G, Rossetti B, Lamonica S, Mondi A, Belmonti S, et al. Switch to raltegravir-based regimens and HIV DNA decrease in patients with suppressed HIV RNA. J Int AIDS Soc. 2014 Jan;17(4 Suppl 3):19791.

114. Lundgren JD, Babiker AG, Gordin F, Emery S, Grund B, Sharma S, et al. Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection. N Engl J Med. 2015 Jul 20;373(9):795–807.

115. Chen YQ, Masse B, Wang L, Ou S-S, Li X, Donnell D, et al. Statistical considerations for the HPTN 052 Study to evaluate the effectiveness of early versus delayed antiretroviral strategies to prevent the sexual transmission of HIV-1 in serodiscordant couples. Contemp Clin Trials. 2012 Nov;33(6):1280–6.

116. Chun T-W, Justement JS, Moir S, Hallahan CW, Maenza J, Mullins JI, et al. Decay of the HIV reservoir in patients receiving antiretroviral therapy for extended periods:

implications for eradication of virus. J Infect Dis. 2007 Jun 15;195(12):1762–4.

117. Jain V, Hartogensis W, Bacchetti P, Hunt PW, Hatano H, Sinclair E, et al. Antiretroviral therapy initiated within 6 months of HIV infection is associated with lower T-cell activation and smaller HIV reservoir size. J Infect Dis. 2013 Oct 15;208(8):1202–11.

118. Ananworanich J, Sacdalan CP, Pinyakorn S, Chomont N, de Souza M, Luekasemsuk T, et al. Virological and immunological characteristics of HIV-infected individuals at the earliest stage of infection. J virus Erad. 2016 Jan;2:43–8.

119. Cao W, Mehraj V, Trottier B, Baril J-G, Leblanc R, Lebouche B, et al. Early Initiation Rather Than Prolonged Duration of Antiretroviral Therapy in HIV Infection Contributes to the Normalization of CD8 T-Cell Counts. Clin Infect Dis. 2016 Jan 15;62(2):250–7.

120. Salgado M, Rabi SA, O'Connell KA, Buckheit RW, Bailey JR, Chaudhry AA, et al. Prolonged control of replication-competent dual- tropic human immunodeficiency virus-1 following cessation of highly active antiretroviral therapy. Retrovirology. 2011 Jan;8:97.

121. Sáez-Cirión A, Bacchus C, Hocqueloux L, Avettand-Fenoel V, Girault I, Lecuroux C, et al. Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study. PLoS Pathog. 2013 Mar;9(3):e1003211.

122. Frange P, Faye A, Avettand-Fenoël V, Bellaton E, Descamps D, Angin M, et al. HIV-1 virological remission lasting more than 12 years after interruption of early antiretroviral therapy in a perinatally infected teenager enrolled in the French ANRS EPF-CO10 paediatric cohort: a case report. lancet HIV. 2016 Jan;3(1):e49–54.

123. Persaud D, Gay H, Ziemniak C, Chen YH, Piatak M, Chun T-W, et al. Absence of detectable HIV-1 viremia after treatment cessation in an infant. N Engl J Med. 2013 Nov 7;369(19):1828–35.

124. Ananworanich J, Robb ML. The transient HIV remission in the Mississippi baby: why is this good news? J Int AIDS Soc. 2014 Jan;17:19859.

125. Luzuriaga K, Gay H, Ziemniak C, Sanborn KB, Somasundaran M, Rainwater-Lovett K, et al. Viremic relapse after HIV-1 remission in a perinatally infected child. N Engl

J Med. 2015 Feb 19;372(8):786–8.

126. Gonzalez SM, Zapata W, Rugeles MT. Role of Regulatory T Cells and Inhibitory Molecules in the Development of Immune Exhaustion During Human Immunodeficiency Virus Type 1 Infection. Viral Immunol. 2015 Jan;29(1):2–10.

127. Abudulai LN, Fernandez S, Corscadden K, Hunter M, Kirkham L-AS, Post JJ, et al. Chronic HIV-1 Infection Induces B-Cell Dysfunction That Is Incompletely Resolved by Long-Term Antiretroviral Therapy. J Acquir Immune Defic Syndr. 2016 Apr 1;71(4):381–9.

128. Molina J-M, Levy Y, Fournier I, Hamonic S, Bentata M, Beck-Wirth G, et al. Interleukin-2 before antiretroviral therapy in patients with HIV infection: a randomized trial (ANRS 119). J Infect Dis. 2009 Jul 15;200(2):206–15.

129. Levy Y, Lacabaratz C, Weiss L, Viard J-P, Goujard C, Lelièvre J-D, et al. Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment. J Clin Invest. 2009 Apr;119(4):997–1007.

130. Sereti I, Dunham RM, Spritzler J, Aga E, Proschan MA, Medvik K, et al. IL-7 administration drives T cell-cycle entry and expansion in HIV-1 infection. Blood. 2009 Jun 18;113(25):6304–14.

131. Lévy Y, Sereti I, Tambussi G, Routy JP, Lelièvre JD, Delfraissy JF, et al. Effects of recombinant human interleukin 7 on T-cell recovery and thymic output in HIV-infected patients receiving antiretroviral therapy: results of a phase I/IIa randomized, placebo-controlled, multicenter study. Clin Infect Dis. 2012 Jul;55(2):291–300.

132. Croce M, Orengo AM, Azzarone B, Ferrini S. Immunotherapeutic applications of IL-15. Immunotherapy. 2012 Sep;4(9):957–69.

133. Pallikkuth S, Micci L, Ende ZS, Iriele RI, Cervasi B, Lawson B, et al. Maintenance of intestinal Th17 cells and reduced microbial translocation in SIV-infected rhesus macaques treated with interleukin (IL)-21. PLoS Pathog. 2013 Jan;9(7):e1003471.

134. Li J, Valentin A, Ng S, Beach RK, Alicea C, Bergamaschi C, et al. Differential effects of IL-15 on the generation, maintenance and cytotoxic potential of adaptive cellular responses induced by DNA vaccination. Vaccine. 2015 Feb 25;33(9):1188–96.

135. Vieillard V, Gharakhanian S, Lucar O, Katlama C, Launay O, Autran B, et al. Perspectives for immunotherapy: which applications might achieve an HIV functional cure? Oncotarget. 2016 Feb 29;

136. DaFonseca S, Chomont N, El Far M, Boulassel R, Routy J, Sékaly R. Purging the HIV-1 reservoir through the disruption of the PD-1 pathway. J Int AIDS Soc. The International AIDS Society; 2010;13(Suppl 3):O15.

137. Porichis F, Kaufmann DE. Role of PD-1 in HIV pathogenesis and as target for therapy. Curr HIV/AIDS Rep. 2012 Mar;9(1):81–90.

138. Xing S, Siliciano RF. Targeting HIV latency: pharmacologic strategies toward eradication. Drug Discov Today. 2013 Jun;18(11-12):541–51.

139. Shan L, Deng K, Shroff NS, Durand CM, Rabi SA, Yang H-C, et al. Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation. Immunity. 2012 Mar 23;36(3):491–501.

140. Mylvaganam GH, Silvestri G, Amara RR. HIV therapeutic vaccines: moving towards a functional cure. Curr Opin Immunol. 2015 Aug;35:1–8.

141. Klatt NR, Shudo E, Ortiz AM, Engram JC, Paiardini M, Lawson B, et al. CD8+ lymphocytes control viral replication in SIVmac239-infected rhesus macaques without decreasing the lifespan of productively infected cells. PLoS Pathog. 2010 Jan;6(1):e1000747.

142. Mueller YM, Do DH, Boyer JD, Kader M, Mattapallil JJ, Lewis MG, et al. CD8+ cell depletion of SHIV89.6P-infected macaques induces CD4+ T cell proliferation that contributes to increased viral loads. J Immunol. 2009 Oct 15;183(8):5006–12.

143. Okoye A, Park H, Rohankhedkar M, Coyne-Johnson L, Lum R, Walker JM, et al. Profound CD4+/CCR5+ T cell expansion is induced by CD8+ lymphocyte depletion but does not account for accelerated SIV pathogenesis. J Exp Med. 2009 Jul 6;206(7):1575–88.

144. Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science. 1999 Feb 5;283(5403):857–60.

145. Deng K, Pertea M, Rongvaux A, Wang L, Durand CM, Ghiaur G, et al. Broad CTL

response is required to clear latent HIV-1 due to dominance of escape mutations. Nature. 2015 Jan 15;517(7534):381–5.

146. Mann JK, Ndung'u T. HIV-1 vaccine immunogen design strategies. Virol J. 2015 Jan;12:3.

147. Ross AL, Bråve A, Scarlatti G, Manrique A, Buonaguro L. Progress towards development of an HIV vaccine: report of the AIDS Vaccine 2009 Conference. Lancet Infect Dis. 2010 May;10(5):305–16.

148. Sandström E, Nilsson C, Hejdeman B, Bråve A, Bratt G, Robb M, et al. Broad immunogenicity of a multigene, multiclade HIV-1 DNA vaccine boosted with heterologous HIV-1 recombinant modified vaccinia virus Ankara. J Infect Dis. 2008 Nov 15;198(10):1482–90.

149. Nilsson C, Aboud S, Karlén K, Marovich M, Wahren B, Sandström E, et al. OA04-02. Strong HIV-specific CD4 and CD8 T-lymphocyte proliferation in HIV-1 DNA prime/modified vaccinia virus Ankara (MVA) heterologous boost vaccinees. Retrovirology. BioMed Central; 2009;6(Suppl 3):O26.

150. Currier J, De Souza M, Ratto-Kim S, Paris R, Nguay V, Cox J, et al. OA04-03. Characterization of cell-mediated immune responses generated by recombinant modified vaccinia Ankara (rMVA)-HIV-1 in a phase I vaccine trial. Retrovirology. BioMed Central; 2009;6(Suppl 3):O27.

151. Borthwick N, Ahmed T, Ondondo B, Hayes P, Rose A, Ebrahimsa U, et al. Vaccine-elicited human T cells recognizing conserved protein regions inhibit HIV-1. Mol Ther. 2014 Feb;22(2):464–75.

152. Hansen SG, Ford JC, Lewis MS, Ventura AB, Hughes CM, Coyne-Johnson L, et al. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. Nature. 2011 May 26;473(7348):523–7.

153. Hansen SG, Piatak M, Ventura AB, Hughes CM, Gilbride RM, Ford JC, et al. Immune clearance of highly pathogenic SIV infection. Nature. 2013 Oct 3;502(7469):100–4.

154. Caskey M, Klein F, Lorenzi JCC, Seaman MS, West AP, Buckley N, et al. Viraemia suppressed in HIV-1-infected humans by broadly neutralizing antibody 3BNC117. Nature. 2015 Jun 25;522(7557):487–91.

155. Halper-Stromberg A, Lu C-L, Klein F, Horwitz JA, Bournazos S, Nogueira L, et al. Broadly neutralizing antibodies and viral inducers decrease rebound from HIV-1 latent reservoirs in humanized mice. Cell. 2014 Aug 28;158(5):989–99.

156. Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell. 1996 Aug 9;86(3):367–77.

157. Paxton WA, Martin SR, Tse D, O'Brien TR, Skurnick J, VanDevanter NL, et al. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure. Nat Med. 1996 Apr;2(4):412–7.

158. Hütter G, Nowak D, Mossner M, Ganepola S, Müssig A, Allers K, et al. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. N Engl J Med. 2009 Feb 12;360(7):692–8.

159. Henrich TJ, Hanhauser E, Marty FM, Sirignano MN, Keating S, Lee T-H, et al. Antiretroviral-free HIV-1 remission and viral rebound after allogeneic stem cell transplantation: report of 2 cases. Ann Intern Med. 2014 Sep 2;161(5):319–27.

160. Martinson JJ, Chapman NH, Rees DC, Liu YT, Clegg JB. Global distribution of the CCR5 gene 32-basepair deletion. Nat Genet. 1997 May;16(1):100–3.

161. Ruiz-Ferrer M, Barroso N, Antiñolo G, Aguilar-Reina J. Analysis of CCR5-Delta 32 and CCR2-V64I polymorphisms in a cohort of Spanish HCV patients using real-time polymerase chain reaction and fluorescence resonance energy transfer technologies. J Viral Hepat. 2004 Jul;11(4):319–23.

162. Petz LD, Burnett JC, Li H, Li S, Tonai R, Bakalinskaya M, et al. Progress toward curing HIV infection with hematopoietic cell transplantation. Stem Cells Cloning. 2015 Jan;8:109–16.

163. Smith AR, Wagner JE. Alternative haematopoietic stem cell sources for transplantation: place of umbilical cord blood. Br J Haematol. 2009 Oct;147(2):246–61.

164. Manjunath N, Yi G, Dang Y, Shankar P. Newer gene editing technologies toward HIV gene therapy. Viruses. 2013 Nov;5(11):2748–66.

165. Tebas P, Stein D, Tang WW, Frank I, Wang SQ, Lee G, et al. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. N Engl J Med. 2014 Mar 6;370(10):901–10.

166. Zhu W, Lei R, Le Duff Y, Li J, Guo F, Wainberg MA, et al. The CRISPR/Cas9 system inactivates latent HIV-1 proviral DNA. Retrovirology. 2015 Jan;12(1):22.

167. Hu W, Kaminski R, Yang F, Zhang Y, Cosentino L, Li F, et al. RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. Proc Natl Acad Sci. 2014 Jul 21;2–7.

168. Kaminski R, Bella R, Yin C, Otte J, Ferrante P, Gendelman HE, et al. Excision of HIV-1 DNA by gene editing: a proof-of-concept in vivo study. Gene Ther. 2016 May 19;[Epub ahead of print].

169. Joseph A, Zheng JH, Follenzi A, Dilorenzo T, Sango K, Hyman J, et al. Lentiviral vectors encoding human immunodeficiency virus type 1 (HIV-1)-specific T-cell receptor genes efficiently convert peripheral blood CD8 T lymphocytes into cytotoxic T lymphocytes with potent in vitro and in vivo HIV-1-specific inhibitory activity. J Virol. 2008 Mar;82(6):3078–89.

170. Nakagawa F, May M, Phillips A. Life expectancy living with HIV: recent estimates and future implications. Curr Opin Infect Dis. 2013 Feb;26(1):17–25.

171. Katlama C, Deeks SG, Autran B, Martinez-Picado J, Van Lunzen J, Rouzioux C, et al. Barriers to a cure for HIV: New ways to target and eradicate HIV-1 reservoirs. Lancet. 2013;381(9883):2109–17.

172. Massanella M, Martinez-Picado J, Blanco J. Attacking the HIV reservoir from the immune and viral perspective. Curr HIV/AIDS Rep. 2013 Mar;10(1):33–41.

173. Recommendations for Testing, Managing, and Treating Hepatitis C [Internet]. [cited 2015 Sep 20]. Available from: http://www.hcvguidelines.org/

174. Asmuth DM, Murphy RL, Rosenkranz SL, Lertora JJL, Kottilil S, Cramer Y, et al. Safety, tolerability, and mechanisms of antiretroviral activity of pegylated interferon Alfa-2a in HIV-1-monoinfected participants: a phase II clinical trial. J Infect Dis. 2010 Jun 1;201(11):1686–96.

175. Tavel J a, Huang C-Y, Shen J, Metcalf J a, Dewar R, Shah A, et al. Interferon-alpha

produces significant decreases in HIV load. J Interferon Cytokine Res. 2010 Jul;30(7):461–4.

176. Manion M, Rodriguez B, Medvik K, Hardy G, Harding C V, Schooley RT, et al. Interferon-alpha administration enhances CD8+ T cell activation in HIV infection. PLoS One. 2012 Jan;7(1):e30306.

177. Pillai SK, Abdel-mohsen M, Guatelli J, Skasko M, Monto A, Fujimoto K. Role of retroviral restriction factors in the interferon- α – mediated suppression of HIV-1 in vivo. Proc Natl Acad Sci U S A. 2012;109(8):3035–40.

178. Torriani FJ, Rodriguez-Torres M, Rockstroh JK, Lissen E, Gonzalez-García J, Lazzarin A, et al. Peginterferon Alfa-2a plus ribavirin for chronic hepatitis C virus infection in HIV-infected patients. N Engl J Med. 2004 Jul 29;351(5):438–50.

179. Neil S, Bieniasz P. Human immunodeficiency virus, restriction factors, and interferon. J Interferon Cytokine Res. 2009 Sep;29(9):569–80.

180. Azzoni L, Foulkes AS, Papasavvas E, Mexas AM, Lynn KM, Mounzer K, et al. Pegylated Interferon alfa-2a monotherapy results in suppression of HIV type 1 replication and decreased cell-associated HIV DNA integration. J Infect Dis. 2013 Jan 15;207(2):213–22.

181. Sun H, Buzon MJ, Shaw A, Berg RK, Yu XG, Ferrando-Martinez S, et al. Hepatitis C therapy with interferon-α and ribavirin reduces CD4 T-cell-associated HIV-1 DNA in HIV-1/hepatitis C virus-coinfected patients. J Infect Dis. 2014 May 1;209(9):1315–20.

182. Jiao Y-M, Weng W-J, Gao Q-S, Zhu W-J, Cai W-P, Li L-H, et al. Hepatitis C therapy with interferon-α and ribavirin reduces the CD4 cell count and the total, 2LTR circular and integrated HIV-1 DNA in HIV/HCV co-infected patients. Antiviral Res. Elsevier B.V.; 2015 Mar 27;118:118–22.

183. Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun. 2004;313(4):856–62.

184. Myers LE, McQuay LJ, Hollinger FB. Dilution assay statistics. J Clin Microbiol. 1994 Mar;32(3):732–9.

185. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem. 2011 Nov 15;83(22):8604–10.

186. Molina-Arcas M, Bellosillo B, Casado FJ, Montserrat E, Gil J, Colomer D, et al. Fludarabine uptake mechanisms in B-cell chronic lymphocytic leukemia. Blood. 2003 Mar 15;101(6):2328–34.

187. Izquierdo-Useros N, Lorizate M, McLaren PJ, Telenti A, Kräusslich H-G, Martinez-Picado J. HIV-1 capture and transmission by dendritic cells: the role of viral glycolipids and the cellular receptor Siglec-1. PLoS Pathog. 2014 Jul;10(7):e1004146.

188. Cheadle C, Fan J, Cho-chung YS, Werner T, Ray J, Do L, et al. Control of gene expression during T cell activation : alternate regulation of mRNA transcription and mRNA stability. 2005;16:1–16.

189. Massanella M, Tural C, Papagno L, Garcia E, Jou A, Bofill M, et al. Changes in T-cell subsets in HIV-HCV-coinfected patients during pegylated interferon-alpha2a plus ribavirin treatment. Antivir Ther. 2010 Jan;15(3):333–42.

190. Singh R, Gaiha G, Werner L, McKim K, Mlisana K, Luban J, et al. Association of TRIM22 with the type 1 interferon response and viral control during primary HIV-1 infection. J Virol. 2011 Jan;85(1):208–16.

191. Kumar A, Zloza A, Moon RT, Watts J, Tenorio AR, Al-Harthi L. Active beta-catenin signaling is an inhibitory pathway for human immunodeficiency virus replication in peripheral blood mononuclear cells. J Virol. 2008 Mar;82(6):2813–20.

192. Henderson LJ, Narasipura SD, Adarichev V, Kashanchi F, Al-Harthi L. Identification of novel T cell factor 4 (TCF-4) binding sites on the HIV long terminal repeat which associate with TCF-4, β-catenin, and SMAR1 to repress HIV transcription. J Virol. 2012 Sep;86(17):9495–503.

193. Rafati H, Lemasters E, Lennert VDD, Boucher C, Vries R, Mahmoudi T. Activation of the Wnt pathway by natural ligands or small molecule inhibitors activates latent HIV. 7th IAS Conference on HIV Pathogenesis, Treatment and Prevention, Kuala Lumpur, Malaysia. 2013.

194. Letendre SL, Woods SP, Ellis RJ, Atkinson JH, Masliah E, van den Brande G, et al.

Lithium improves HIV-associated neurocognitive impairment. AIDS. 2006 Sep 11;20(14):1885–8.

195. Schifitto G, Zhong J, Gill D, Peterson DR, Gaugh MD, Zhu T, et al. Lithium therapy for human immunodeficiency virus type 1-associated neurocognitive impairment. J Neurovirol. 2009 Apr;15(2):176–86.

196. Xue H-H, Zhao D-M. Regulation of mature T cell responses by the Wnt signaling pathway. Ann N Y Acad Sci. 2012 Jan;1247:16–33.

197. Kulpa DA, Brehm JH, Fromentin R, Cooper A, Cooper C, Ahlers J, et al. The immunological synapse: the gateway to the HIV reservoir. Immunol Rev. 2013 Jul;254(1):305–25.

198. Yukl SA, Shergill AK, Ho T, Killian M, Girling V, Epling L, et al. The distribution of HIV DNA and RNA in cell subsets differs in gut and blood of HIV-positive patients on ART: implications for viral persistence. J Infect Dis. 2013 Oct 15;208(8):1212–20.

199. Rasmussen T, Tolstrup M, Brinkmann C, Olesen R, Erikstrup C, Solomon A, et al. Cyclic dosing of panobinostat to reverse HIV-latency: Findings from a clinical trial. 6th International Workshop on HIV Persistence during Therapy Miami, FL, USA. 2013. p. Abstract 53.

200. Margolis D, Archin N, Kuruc J, Bateson R, Dahl N, Crooks A, et al. Translational Challenges in Targeting Latent HIV Infection. 6th International Workshop on HIV Persistence during Therapy Miami, FL, USA. 2013. p. Abstract 51.

201. Noto A, Trautmann L. Developing Combined HIV Vaccine Strategies for a Functional Cure. Vaccines. 2013 Jan;1(4):481–96.

202. Archin NM, Margolis DM. Emerging strategies to deplete the HIV reservoir. Curr Opin Infect Dis. 2014 Mar;27(1):29–35.

203. Casazza JP, Bowman KA, Adzaku S, Smith EC, Enama ME, Bailer RT, et al. Therapeutic vaccination expands and improves the function of the HIV-specific memory T-cell repertoire. J Infect Dis. 2013 Jun 15;207(12):1829–40.

204. Achenbach CJ, Till M, Palella FJ, Knoll MD, Terp SM, Kalnins AU, et al. Extended antiretroviral treatment interruption in HIV-infected patients with long-term

suppression of plasma HIV RNA. HIV Med. 2005 Jan;6(1):7–12.

205. Li JZ, Brumme ZL, Brumme CJ, Wang H, Spritzler J, Robertson MN, et al. Factors associated with viral rebound in HIV-1-infected individuals enrolled in a therapeutic HIV-1 gag vaccine trial. J Infect Dis. 2011 Apr 1;203(7):976–83.

206. García F, Lejeune M, Climent N, Gil C, Alcamí J, Morente V, et al. Therapeutic immunization with dendritic cells loaded with heat-inactivated autologous HIV-1 in patients with chronic HIV-1 infection. J Infect Dis. 2005 May 15;191(10):1680–5.

207. Pollard RB, Rockstroh JK, Pantaleo G, Asmuth DM, Peters B, Lazzarin A, et al. Safety and efficacy of the peptide-based therapeutic vaccine for HIV-1, Vacc-4x: a phase 2 randomised, double-blind, placebo-controlled trial. Lancet Infect Dis. 2014 Apr;14(4):291–300.

208. Mothe B, Climent N, Plana M, Rosàs M, Jiménez JL, Muñoz-Fernández MÁ, et al. Safety and immunogenicity of a modified vaccinia Ankara-based HIV-1 vaccine (MVA-B) in HIV-1-infected patients alone or in combination with a drug to reactivate latent HIV-1. J Antimicrob Chemother. 2015 Jan;70(6):1833–42.

209. Kinloch-de Loes S, Hoen B, Smith DE, Autran B, Lampe FC, Phillips AN, et al. Impact of therapeutic immunization on HIV-1 viremia after discontinuation of antiretroviral therapy initiated during acute infection. J Infect Dis. 2005 Aug 15;192(4):607–17.

210. Létourneau S, Im E-J, Mashishi T, Brereton C, Bridgeman A, Yang H, et al. Design and pre-clinical evaluation of a universal HIV-1 vaccine. PLoS One. 2007 Jan;2(10):e984.

211. Ternette N, Block PD, Sánchez-Bernabéu Á, Borthwick N, Pappalardo E, Abdul-Jawad S, et al. Early Kinetics of the HLA Class I-Associated Peptidome of MVA.HIVconsv-Infected Cells. J Virol. 2015 Jun;89(11):5760–71.

212. Liu MKP, Hawkins N, Ritchie AJ, Ganusov V V, Whale V, Brackenridge S, et al. Vertical T cell immunodominance and epitope entropy determine HIV-1 escape. J Clin Invest. 2013 Jan;123(1):380–93.

213. Dorrell L, Yang H, Ondondo B, Dong T, di Gleria K, Suttill A, et al. Expansion and diversification of virus-specific T cells following immunization of human immunodeficiency virus type 1 (HIV-1)-infected individuals with a recombinant

modified vaccinia virus Ankara/HIV-1 Gag vaccine. J Virol. 2006 May;80(10):4705–16.

214. Yang H, Wu H, Hancock G, Clutton G, Sande N, Xu X, et al. Antiviral inhibitory capacity of CD8+ T cells predicts the rate of CD4+ T-cell decline in HIV-1 infection. J Infect Dis. 2012 Aug 15;206(4):552–61.

215. Laird NM, Ware JH. Random-effects models for longitudinal data. Biometrics. 1982 Dec;38(4):963–74.

216. Lindstrom MJ, Bates DM. Newton-Raphson and EM Algorithms for Linear Mixed-Effects Models for Repeated-Measures Data. J Am Stat Assoc. 1988;83(404):1014–22.

217. Greenough TC, Cunningham CK, Muresan P, McManus M, Persaud D, Fenton T, et al. Safety and immunogenicity of recombinant poxvirus HIV-1 vaccines in young adults on highly active antiretroviral therapy. Vaccine. 2008 Dec 9;26(52):6883–93.

218. Harrer E, Bäuerle M, Ferstl B, Chaplin P, Petzold B, Mateo L, et al. Therapeutic vaccination of HIV-1-infected patients on HAART with a recombinant HIV-1 nef-expressing MVA: safety, immunogenicity and influence on viral load during treatment interruption. Antivir Ther. 2005 Jan;10(2):285–300.

219. Gasser O, Brander C, Wolbers M, Brown N V, Rauch A, Günthard HF, et al. Expansion of interferon-γ-secreting HIV-specific T cells during successful antiretroviral therapy. HIV Med. 2013 Apr;14(4):241–6.

220. Mothe B, Manzardo C, Coll P, Sanchez A, Escrig R. Safety and immunogenicity of ChAd.HIVconsv and MVA.HIVconsv therapeutic vaccines in a cohort of early treated HIV-1 infected individuals. 8th International AIDS Society Conference HIV Pathogenesis, Treatment and Prevention, Vancouver. 2015. p. Abstract 2474.

221. Hancock G, Yang H, Yorke E, Wainwright E, Bourne V, Frisbee A, et al. Identification of Effective Subdominant Anti-HIV-1 CD8+ T Cells Within Entire Post-infection and Post-vaccination Immune Responses. PLoS Pathog. 2015 Feb;11(2):e1004658.

222. Mothe B, Llano A, Ibarrondo J, Daniels M, Miranda C, Zamarreño J, et al. Definition of the viral targets of protective HIV-1-specific T cell responses. J Transl Med. 2011 Jan;9:208.

223. Kunwar P, Hawkins N, Dinges WL, Liu Y, Gabriel EE, Swan DA, et al. Superior control of HIV-1 replication by CD8+ T cells targeting conserved epitopes: implications for HIV vaccine design. PLoS One. 2013 Jan;8(5):e64405.

224. Rihn SJ, Wilson SJ, Loman NJ, Alim M, Bakker SE, Bhella D, et al. Extreme Genetic Fragility of the HIV-1 Capsid. Luban J, editor. PLoS Pathog. 2013 Jun 20;9(6):e1003461.

225. Rolland M, Manocheewa S, Swain JV, Lanxon-Cookson EC, Kim M, Westfall DH, et al. HIV-1 conserved-element vaccines: relationship between sequence conservation and replicative capacity. J Virol. 2013 May;87(10):5461–7.

226. Mothe B, Hu X, Llano A, Rosati M, Olvera A, Kulkarni V, et al. A human immune data-informed vaccine concept elicits strong and broad T-cell specificities associated with HIV-1 control in mice and macaques. J Transl Med. 2015 Jan;13:60.

227. Thiele F, Tao S, Zhang Y, Muschaweckh A, Zollmann T, Protzer U, et al. Modified vaccinia virus Ankara-infected dendritic cells present CD4+ T-cell epitopes by endogenous major histocompatibility complex class II presentation pathways. J Virol. 2015 Mar;89(5):2698–709.

228. Novis CL, Archin NM, Buzon MJ, Verdin E, Round JL, Lichterfeld M, et al. Reactivation of latent HIV-1 in central memory $CD4^{+}$ T cells through TLR-1/2 stimulation. Retrovirology. 2013 Jan;10:119.

229. Matsumiya M, Stylianou E, Griffiths K, Lang Z, Meyer J, Harris SA, et al. Roles for Treg expansion and HMGB1 signaling through the TLR1-2-6 axis in determining the magnitude of the antigen-specific immune response to MVA85A. PLoS One. 2013 Jan;8(7):e67922.

230. Graf EH, Pace MJ, Peterson BA, Lynch LJ, Chukwulebe SB, Mexas AM, et al. Gag-positive reservoir cells are susceptible to HIV-specific cytotoxic T lymphocyte mediated clearance in vitro and can be detected in vivo [corrected]. PLoS One. 2013 Jan;8(8):e71879.

231. Achenbach CJ, Assoumou L, Deeks SG, Wilkin TJ, Berzins B, Casazza JP, et al. Effect of therapeutic intensification followed by HIV DNA prime and rAd5 boost vaccination on HIV-specific immunity and HIV reservoir (EraMune 02): a

multicentre randomised clinical trial. lancet HIV. 2015 Mar;2(3):e82–91.

232. Walker-Sperling VEK, Cohen VJ, Tarwater PM, Blankson JN. Reactivation Kinetics of HIV-1 and Susceptibility of Reactivated Latently Infected CD4+ T Cells to HIV-1-Specific CD8+ T Cells. J Virol. 2015 Sep;89(18):9631–8.

233. Ternette N, Yang H, Partridge T, Llano A, Cedeño S, Fischer R, et al. Defining the HLA class I-associated viral antigen repertoire from HIV-1-infected human cells. Eur J Immunol. 2016 Jan;46(1):60–9.

234. Sáez-Cirión A, Lacabaratz C, Lambotte O, Versmisse P, Urrutia A, Boufassa F, et al. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. Proc Natl Acad Sci U S A. 2007 Apr 17;104(16):6776–81.

235. Buzon MJ, Yang Y, Ouyang Z, Sun H, Seiss K, Rogich J, et al. Susceptibility to CD8 T-cell-mediated killing influences the reservoir of latently HIV-1-infected CD4 T cells. J Acquir Immune Defic Syndr. 2014 Jan 1;65(1):1–9.

236. Allers K, Hütter G, Hofmann J, Loddenkemper C, Rieger K, Thiel E, et al. Evidence for the cure of HIV infection by CCR5Δ32/Δ32 stem cell transplantation. Blood. 2011 Mar 10;117(10):2791–9.

237. Henrich TJ, Hu Z, Li JZ, Sciaranghella G, Busch MP, Keating SM, et al. Long-term reduction in peripheral blood HIV type 1 reservoirs following reduced-intensity conditioning allogeneic stem cell transplantation. J Infect Dis. 2013;207(11):1694–702.

238. Deeks SG, McCune JM. Can HIV be cured with stem cell therapy? Nat Biotechnol. 2010 Aug;28(8):807–10.

239. Zaia JA, Forman SJ. Transplantation in HIV-infected subjects: is cure possible? Hematology Am Soc Hematol Educ Program. 2013 Jan;2013:389–93.

240. Hütter G, Thiel E. Allogeneic transplantation of CCR5-deficient progenitor cells in a patient with HIV infection: an update after 3 years and the search for patient no. 2. AIDS. 2011 Jan 14;25(2):273–4.

241. Kordelas L, Verheyen J, Beelen DW, Horn PA, Heinold A, Kaiser R, et al. Shift of HIV tropism in stem-cell transplantation with CCR5 Delta32 mutation. N Engl J

Med. 2014 Aug 28;371(9):880–2.

242. Verheyen J, Esser S, Kordelas L. More on shift of HIV tropism in stem-cell transplantation with CCR5 delta32/delta32 mutation. N Engl J Med. 2014 Dec 18;371(25):2438.

243. Lee SJ, Klein J, Haagenson M, Baxter-Lowe LA, Confer DL, Eapen M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. Blood. 2007 Dec 15;110(13):4576–83.

244. Ballen KK, Gluckman E, Broxmeyer HE. Umbilical cord blood transplantation: the first 25 years and beyond. Blood. 2013 Jul 25;122(4):491–8.

245. Gonzalez G, Park S, Chen D, Armitage S, Shpall E, Behringer R. Identification and frequency of CCR5Δ32/Δ32 HIV-resistant cord blood units from Houston area hospitals. HIV Med. 2011 Sep;12(8):481–6.

246. Petz LD, Redei I, Bryson Y, Regan D, Kurtzberg J, Shpall E, et al. Hematopoietic cell transplantation with cord blood for cure of HIV infections. Biol Blood Marrow Transplant. 2013 Mar;19(3):393–7.

247. Alizadeh M, Bernard M, Danic B, Dauriac C, Birebent B, Lapart C, et al. Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. Blood. 2002 Jun 15;99(12):4618–25.

248. Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, et al. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature. 1996 Aug 22;382(6593):722–5.

249. Lengauer T, Sander O, Sierra S, Thielen A, Kaiser R. Bioinformatics prediction of HIV coreceptor usage. Nat Biotechnol. 2007 Dec;25(12):1407–10.

250. Blankson JN, Bailey JR, Thayil S, Yang H-C, Lassen K, Lai J, et al. Isolation and characterization of replication-competent human immunodeficiency virus type 1 from a subset of elite suppressors. J Virol. 2007 Mar;81(5):2508–18.

251. Liu TF, Shafer RW. Web resources for HIV type 1 genotypic-resistance test interpretation. Clin Infect Dis. 2006 Jun 1;42(11):1608–18.

252. Salgado M, Swanson MD, Pohlmeyer CW, Buckheit RW, Wu J, Archin NM, et al. HLA-B*57 elite suppressor and chronic progressor HIV-1 isolates replicate vigorously and cause CD4+ T cell depletion in humanized BLT mice. J Virol. 2014 Mar;88(6):3340–52.

253. Magro E, Regidor C, Cabrera R, Sanjuán I, Forès R, Garcia-Marco JA, et al. Early hematopoietic recovery after single unit unrelated cord blood transplantation in adults supported by co-infusion of mobilized stem cells from a third party donor. Haematologica. 2006 May;91(5):640–8.

254. Sánchez-Ortega I, Arnan M, Patiño B, Herrero MJ, Querol S, Duarte RF. Early engraftment and full-donor chimerism after single-cord blood plus third-party donor dual transplantation in patients with high-risk acute leukemia. Bone Marrow Transplant. 2014 Jan;49(1):145–7.

255. Kwon M, Bautista G, Balsalobre P, Sánchez-Ortega I, Serrano D, Anguita J, et al. Haplo-cord transplantation using CD34+ cells from a third-party donor to speed engraftment in high-risk patients with hematologic disorders. Biol Blood Marrow Transplant. 2014 Dec;20(12):2015–22.

256. Paquette J. U of M researchers conduct world's first cord blood transplant aimed at curing Leukemia and HIV/AIDS [Internet]. (accessed May 9, 2016). Available from: http://www.healthtalk.umn.edu/2013/04/23/u-of-m-researchers-conduct-worlds-first-cord-blood-transplant-aimed-at-curing-leukemia-and-hivaids/

257. Kuball JH, Ellerbroek P, Balsalobre P, Serrano D, Buno I, Gayoso J, et al. Single Cord Blood Transplantation Combined With An HLA Mismatched Third Party Donor For High-Risk Hematological Patients With HIV Infection. Blood. American Society of Hematology; 2013 Nov 15;122(21):3401.

258. Agrawal L, Lu X, Qingwen J, VanHorn-Ali Z, Nicolescu IV, McDermott DH, et al. Role for CCR5Delta32 protein in resistance to R5, R5X4, and X4 human immunodeficiency virus type 1 in primary CD4+ cells. J Virol. 2004 Mar;78(5):2277–87.

259. Duarte R, Labopin M, Badoglio M, Al. E. Allogeneic transplantation in patients with HIV-infection: a pair matched cohort study by the European Society for Blood and Marrow Transplantation. Bone marrow transplantation. 2015. p. Abstract 007. 50(suppl 1):S5–6.

260. Sureda A, Bader P, Cesaro S, Dreger P, Duarte RF, Dufour C, et al. Indications for allo- and auto-SCT for haematological diseases, solid tumours and immune disorders: current practice in Europe, 2015. Bone Marrow Transplant. 2015 Aug;50(8):1037–56.

261. Cillo AR, Mellors JW. Which therapeutic strategy will achieve a cure for HIV-1? Curr Opin Virol. Elsevier B.V.; 2016;18:14–9.

262. Gruell H, Klein F. Opening Fronts in HIV Vaccine Development: Tracking the development of broadly neutralizing antibodies. Nat Med. 2014 May;20(5):478–9.

263. Herrera-Carrillo E, Berkhout B. Bone Marrow Gene Therapy for HIV/AIDS. Viruses. 2015 Jul;7(7):3910–36.

CHAPTER 7. PUBLICATIONS

---

1. Maria C. Puertas*, Maria Salgado*, Sara Morón-López, Dan Ouchi, Jose A. Muñoz-Moreno, José Moltó, Bonaventura Clotet and Javier Martinez-Picado. ***Effect of lithium on HIV-1 expression and proviral reservoir size in the $CD4^{+}$ T cells of antiretroviral therapy suppressed patients.*** AIDS, 2014.

2. Rafael F. Duarte, María Salgado, Isabel Sánchez-Ortega, Montserrat Arnan, Carmen Canals, Eva Domingo-Domenech, Alberto Fernández-de-Sevilla, Eva González-Barca, Sara Morón-López, Nuria Nogues, Beatriz Patiño, Maria Carmen Puertas, Bonaventura Clotet, Lawrence D. Petz, Sergio Querol, Javier Martinez-Picado. ***CCR5 Δ32 homozygous cord blood allogeneic transplantation in a patient with HIV: a case report.*** Lancet HIV, 2015.

3. Sara Morón-López, Elisabet Gómez-Mora, Maria Salgado, Dan Ouchi, Maria C. Puertas, Víctor Urrea, Jordi Navarro, Antoni Jou, Mercedes Pérez, Cristina Tural, Bonaventura Clotet, Luis J. Montaner, Julià Blanco, Manuel Crespo, Javier Martinez-Picado. ***Short-term treatment with interferon alpha diminishes expression of HIV-1 and reduces CD4+ T-cell activation in patients coinfected with HIV and hepatitis C virus and receiving antiretroviral therapy.*** Journal Infectious Diseases, 2016.

4. Gemma Hancock*, Sara Morón-López*, Maria C. Puertas, Eleni Giannoulatou, Annie Rose, Maria Salgado, Emma-Jo Hayton, Catharine Morgan, Brian Angus, Fabian Chen, Hongbing Yang, Javier Martinez-Picado, Tomas Hanke, Lucy Dorrell. ***Evaluation of the immunogenicity and impact on the latent HIV-1 reservoir of a conserved region vaccine, MVA.HIVconsv, in antiretroviral therapy-treated subjects.*** Submitted.

5. Marta Martinez-Bonet, Alejandro Gonzalez-Serna, Maria Isabel Clemente, Sara Morón-López, Laura Díaz, Marisa Navarro, Maria C. Puertas, Manuel Leal, Ezequiel Ruiz-Mateos, Javier Martinez-Picado, Maria Angeles Muñoz-Fernandez. ***Relationship between CCR5(Δ32/WT) heterozygosity and HIV-1 reservoir size in adolescents and young adults with perinatally acquired HIV-1 infection.*** Submitted

CHAPTER 8. ACKNOWLEDGEMENTS

Bueno, qué más me queda por explicaros… ¡Ah sí, la parte más importante! El gran esperado capítulo de agradecimientos, que suele ser lo primero que compañeros, amigos y familiares van a leer, porque del resto ya han sido informados a lo largo de la tesis (con más o menos detalle…). Tesis que han vivido, disfrutado, batallado, y en ocasiones sufrido, pero sobre todo apoyado tanto como uno mismo.

Como en toda tesis, ha habido muchas personas implicadas, sin las cuales este trabajo no habría podido realizarse.

En primer lugar, querría agradecer a **Javier** que me diese la oportunidad de formar parte de su grupo como estudiante de doctorado, pese a que en la entrevista sólo supe formular el perclorato potásico ($KClO_4$), pero no así la D-glucosa (siempre se me dio mejor la química inorgánica…). Gracias también porque no fue fácil conseguir beca para poder empezar el doctorado, pero aún y ser difícil, nada es imposible y tras enviar recursos administrativos, ¡ahí estaba mi ansiada beca de doctorado de 3 años! Y, por increíble que parezca en 3 años SÍ se puede hacer un doctorado :D Mil gracias por cómo eres como jefe, por cómo has sido como director de tesis, y por saber transmitir toda tu experiencia, conocimiento y apoyo.

Y ahora tocan **¡¡¡l@s GREC@S!!!** ¡Empiezo por el RESERVOIR TEAM! **Mari Carmen**, muchas gracias por ser una perfecta Lab Manager! Pero también por descubrirme la amada y odiada, por igual, droplet digital PCR; por compartir tu experiencia, sabiduría y consejo, tanto laboral como personal, pero sobre todo gracias por todos los momentos compartidos tanto en Irsi como fuera. **María** (con acento!) **S**, muchísimas gracias por enseñarme el *viral outgrowth assay* y transmitirme todo tu conocimiento sobre reservorios y secuenciación, pero sobre todo gracias por ser como eres, por siempre estar con una sonrisa y de buen humor, que, pese a ser hiperactiva y estar estresada, nunca pierdes. Gracias a las dos por todo vuestro apoyo y por guiarme estos 3 años y poco, sin vosotras todo este trabajo no habría sido posible. **Cristina** (Gálvez, nuestra Cris!), la sucesora pre-Doc del reservoir team, muchas gracias por tu apoyo y ayuda, tus ay qué susto, tu risa pegadiza y tus miradas que matan sin querer, jajaja; te deseo todo lo mejor en este camino que inicias hacia tu tesis. Y ahora vamos con el ¡DENDRITIC

8

TEAM! **Nuria**, gracias por todos tus consejos, por enseñar que si uno mima a las células siempre crecen mejor y por intentar siempre que las DCs y Siglec-1 se puedan asociar con cualquier tema, si uno no lo plantea seguro nunca pasa. Mil gracias también por tu apoyo en mi primera presentación oral en el Keystone de Boston. **Itziar**, muchísimas gracias por tu ayuda y transmitir tu sabiduría en citometría; pero sobre todo gracias por tu paciencia y rápida respuesta a: "el RUPPER no va, no sé que le pasa pero no adquiere… :S (icono pánico, miedo y terror)". **Maria Pino**, la nostra més recent Doctora!!!! Moltes felicitaaats de nou!!!! I moltes gràcies per respondre tots els meus dubtes pre-tesi, tant administratius com de format. Ha sigut un plaer compartir espai de treball i grup durant els nostres doctorats, i també un plaer que ens hagis encomanat la teva passió pel swing. Et desitjo molta sort en la nova etapa que inicies!!! ☺ **Susana**, la nostra metgessa i Doctora Benet!! Jejeje. Moltes gràcies per tota la teva ajuda, tant al laboratori com en l'àmbit mèdic, i gràcies també per sempre tenir un somriure per tothom, perquè ens omples d'energia. **Dani**, nuestro vidente personal, horoscopista particular, actor, bailarín, cocinero/futuro chef, eres el artista del grupo!!! Muchas gracias por alegrarnos cada día con el horoscope time y por tantas charlas sobre series y pelis (sobre todo de temática de terror y suspense, porque sabes que nunca olvidaremos "It follows", y los pasillos de Can Ruti no ayudan…). **María Teresa**, muchas gracias por toda tu ayuda y apoyo durante mi inicio y tu final de doctorado, y también por continuar en contacto aunque estés en tierras londinenses. Aunque debo avergonzarme porque no he podido continuar con tu nivel las dosis frikis semanales, no consigo encontrar algo friki para cada semana T.T ¡¡¡Y no puede faltar la ESTADÍSTICA!!! **Dan**, gracias por haberme enseñado gran parte de los conocimientos que tengo de estadística y por batallar juntos con los datos de IFN, se te echa de menos en Irsi… **Víctor**, nuestro abstemio del grupo pese a que nos empecinemos en quererte emborrachar regalándote libros sobre vinos y botellas de vino/licor en el amigo invisible. Mil gracias por continuar enseñándome conceptos estadísticos, tanto en las cápsulas estadísticas de las reuniones de GREC como cuando te incordio con preguntas en los módulos, y también gracias por tu paciencia al explicárnoslo, porque siempre sabes cuándo nos hemos enterado y cuándo no, y nos lo vuelves a explicar. **Maria Francesca**, nuestra post-doc más reciente que nos ha traído "aires di Italia", gracias por com-

partir nuestras experiencias de aero-combat y mil gracias por ser como eres simpática y extrovertida. Y, por último, aunque no menos importante… **Judith**, nuestra Project Manager, gestora de cohortes y solicitudes al CEIC, muchas gracias por toda tu gestión en el grupo y por tu paciencia siempre que voy a preguntarte algo (que soy bastante cansina, lo sé). Mil gracias a tod@s porque ha sido un gran placer haber realizado la tesis con vosotr@s como compañer@s de grupo.

Y ahora continuamos con el resto de grupos.

En primer lugar querría agradecer todo el trabajo realizado por el **Servicio de procesamiento de muestras**: **Rafi, Lucía y Eulàlia**, porque sin vosotras los estudios clínicos serían prácticamente imposibles de realizar y porque sois un Sol como personas.

Luego toca el turno de l@s **VICs, VITIs** y **AlbaJun@s**. Gracias por hacer que sea tan fácil trabajar con vosotros. Primer, gràcies **Julià**, perquè ha sigut i és un plaer col·laborar en la realització de projectes i gràcies també per compartir la teva experiència i coneixements. **Eli Gómez**, mil gràcies perquè també ha sigut un plaer treballar conjuntament i per transmetre'm molts dels teus coneixements de citometria, però sobretot, gràcies per la teva simpatia i amabilitat. Ànims!!!!!, que la meta ja es veu al final del camí ;). **Luís**, ¡¡¡¡el próximo Doctor de Irsi!!!! Ya casi lo tienes :D Muchas gracias por tu paciencia al responder todas mis dudas administrativas, tanto de becas como del visado de USA, incluso estando a miles de kilómetros. Mucha suerte en la nueva etapa que iniciarás en breve como PhD. **Lucía**, la brisa de aire fresco que tenemos en Irsi, siempre consigues animar a cualquiera, muchas gracias por todo. **Marisa**, muchísimas gracias por tu amabilidad y simpatía, por todos los desayunos compartidos, y los "vamos a comer?", "cómo va por P3?"… **Jorge**, gracias por todos los momentos compartidos y por los "te queda mucho?" cuando ya casi no queda nadie en los módulos, por compartir tus conocimientos y hacer que casi cada día me vaya a dormir sabiendo algo nuevo. ¡Eres el libro de petete! **Eli García**, gracias por las charlas sobre pelis y series de terror, siempre es agradable encontrar a alguien que comparte gustos cinéfilos. **Ferran**, gracias por el placer de haber trabajado contigo durante casi 3 años y por el año compartido de máster. **Ceci**, gracias, pese a que trabajar en molecular a veces sea un poco suplicio por el calor, sabemos que eres friolera… ;) Y mil gra-

cias a **Silvia**, y los recientemente incorporados **Cristina Lorca**, **Ester Aparicio**, **Francesc**, **Montse** y **Ferran**.

Ahora **VIRIEVAC**. **Julia**, muchas gracias por todo. Por ser vecina de mesa en los módulos, por compartir todo tu conocimiento y consejo conmigo, y por seguir mi evolución a lo largo de la tesis. Fue un placer también compartir el GeSIDA de Málaga y el congreso de persistencia de Miami. **Alba**, gràcies per la teva amabilitat i simpatia. Vull que sàpigues que encara em sap greu el “toque” que vam tenir amb els cotxes ☹ poc abans que llegissis la teva tesi (espero que no em torni a passar ara que em queda poc a mi... :S). ¡¡¡Suerte a las dos en el oral del HIV Cure!!! ¡Os irá genial! Y, **Esther Jiménez** y **Ruth**, mil gracias porque es un placer compartir P3 con vosotras. Y a **Hari**, por los meses compartidos durante el máster y el viaje compartido a NY y Boston.

Gracias también al **grupo de Christian Brander**, porque he tenido el placer de colaborar con ellos en varios proyectos. **Bea Mothe**, **Àlex** i **Míriam**, moltes gràcies per tot, però sobretot perquè va ser una molt bona experiència compartir amb vosaltres el meu primer congrés internacional a Cape Town. Muchas gracias también al resto de “branders”, **Marta Ruíz, Marta Marszalek, Sandra Silva, Pep Coll, Anuska y Samandhy**, porque es un placer compartir espacio de trabajo con vosotr@s.

Y cómo no, gracias también a los **grupos de Roger Paredes, Miguel Ángel y José Esté**. **Maria Casadellà**, moltes gràcies i ànims en l’sprint final en la cursa del doctorat, ja ho tens gairebé enllestit!!! **Christian Pou** (malgrat compartir pocs mesos a Irsi) gràcies pel teu humor i les teves bromes amb les que animaves i segur continues animant a qualsevol. **Sandra Franco**, gràcies per com amb l’Eli compartir xerrades sobre pelis i sèries de por. **Maria Nevot**, gràcies per compartir el congrés de GeSIDA de Màlaga i el superviatge de tornada en tren a les tantes de la nit camí de Mollet i Granollers. **Roger Badia**, gràcies per les xerrades sobre motos i sobre The Walking Dead, sempre és un plaer treballar amb tu al costat a P3, tot i que sempre intentis prendre’ns el pèl xD. También gracias a **Cristina Rodríguez**, **Mariona, Yolanda, Javier Rivera, Marc, Muntsa, Ana, Ester Ballana, Eva y Maria Pujantell**.

Y por supuesto, gracias a **Servicios**, **Teresa y Cristina Ramírez**, por todas las secuenciaciones que os he pedido. A **RIMO**, **Chiara, Mireia y,** nuevamente**, Judith**, por vuestra gestión administrativa de proyectos, becas y otras muchas cosas que se me escapan. A **Lidia**, por su gestión como Lab Manager general de Irsi. A **Penélope, Cristina Mesa y Arnau**, por vuestra eficiente gestión administrativa y por vuestra ayuda al gestionar facturas, pedidos, etc. A los **médicos** implicados en todos los estudios clínicos realizados, tanto de la Fundació Lluita contra la SIDA (**José A. Muñoz, José Moltó, Toni Jou y Cristina Tural**) como de la Vall d'Hebron (**Manel Crespo y Jordi Navarro**) y de Bellvitge; y por supuesto, a **enfermer@s** y **monitor@s** de los ensayos. També moltes gràcies, a l'equip de **Comunicació**, **Júlia, Rosina i Josep**, perquè feu una gran feina de divulgació científica que és essencial per a poder transmetre el avenços en recerca que realitzem. Mil gracias también a **Julián**, por resolver todas las dudas informáticas que se nos plantean y gestionar los datos de Irsi. Gracias **Lourdes**, por toda la gestión burocrática y administrativa que realizas como gerente de Irsi. I, sobretot gràcies a tu **Ventura**, perquè IrsiCaixa es va poder formar i continua generant dia rere dia dades molt rellevants en el camp de la investigació en VIH gràcies a la teva gestió i organització. Gracias a tod@s vosotr@s y a l@s que en algún momento habéis pasado por Irsi, ya sea como becari@s de grado, máster o estancia de doctorado, porque de alguna u otra manera habéis contribuido a la realización de esta tesis.

Moreover, I would like to thank some people I had the pleasure to work with. **Lucy Dorrell** and **Gemma Hancock**, it was a pleasure to collaborate with you and to learn a lot about vaccines and immune responses.

Y porque, aunque parezca mentira, existe vida fuera de P3. Querría agradecer todo el apoyo a mis amig@s. **Guille**, porque no perdemos el contacto pese a que estás hecho casi un completo nórdico. **Leire**, mil gracias por tu apoyo y participación en la corrección de esta tesis, pero sobre todo por conseguir mantener el contacto aunque estemos a miles de quilómetros. **Sonia**, gracias porque pese a que nos vemos poco, siempre es como si no pasara el tiempo. **David y Jesús**, muchas gracias por vuestro apoyo durante estos años de tesis, y sobre todo, David, también gracias por tu colaboración en la corrección de esta tesis. Es un placer tener amig@s como vosotr@s.

También quiero agradecer a **mis padres**, **Carmen y José Antonio**, todo su apoyo durante toda mi vida, no sólo durante esta tesis, ya que sin su ayuda y esfuerzo durante los 27 años que tengo, nunca habría podido llegar a ser la persona que soy y a poder hacer realidad mis sueños. Gracias a mis tíos, **Josefina y José Antonio**, porque siempre han sido, son y serán unos segundos padres para mí. Mil gracias a mis yayos, **Francisco, Antonio y Cristina**, porque seguro estarían muy orgullosos; y a mi yaya **Salud**, porque sé que está muy orgullosa. Muchas gracias también a mi hermana, **Estefanía**, y a mi casi hermano, **Carlos** (que te conozco desde hace más de media vida), por todos los momentos compartidos y vuestro continuo apoyo; y a la preciosa hija que tenéis, mi sobrinilla **Lucía**, porque me llena de energía y vitalidad cada día. Esta peque que quiere ser Doctora como su tita Sara, aunque no entienda muy bien todavía porqué ella ya ha pasado a P4 y yo todavía sigo en P3. Gracias también a mi hermano y su mujer, **Ricardo y Ana**, y a mis sobrinos, **Alejandro y Óscar**, por compartir estos años juntos. Gràcies també al meu cunyat, en **Jordi**, per la seva participació en aquesta tesi, i la seva xicota, la **Laura**; i també als meus sogres, en **Jordi i** la **Dolors**, és un plaer compartir la meva vida amb vosaltres.

Gracias también a ti, **Àlex**, mi compañero de viaje en este camino. Moltes gràcies per tots els sucs de taronja i per preparar-me cada matí l'esmorzar que m'enduc a Irsi. També gràcies pel teu recolzament incondicional, i per tots els "hey ho, lets go!" i el que signifiquen. No sé què ens depararà el futur, però sigui on sigui, "it will always be a good time!".

Y por último, aunque uno de los agradecimientos más importantes. Gracias a todos los **pacientes** que han participado en los estudios clínicos recogidos en esta tesis, porque sin su altruista colaboración la investigación no podría avanzar. Mil gracias y seguiremos luchando porque un día se pueda conseguir la cura contra la infección por VIH.

Simplemente, ¡GRACIAS!