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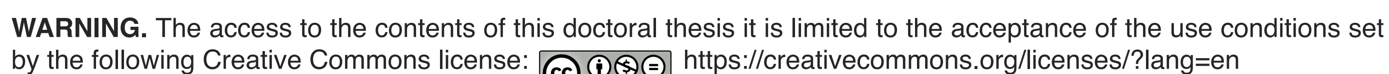
WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

Departament de Biologia Cel·lular, Fisiologia i d'Immunologia Facultat de Medicina

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Challenges and prospects for an immune-driven functional cure of HIV infection

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Memòria de la tesi presentada per obtenir el grau de Doctora en Immunologia Avançada per la Universitat Autònoma de Barcelona, 2019

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Dr. Christian Brander Dra. Beatriz Mothe Pujadas

ABBREVIATIONS

2-LTR	two-long terminal repeat
7-AAD	7-Aminoactinomycin D
acH3	Histone H3 acetylation
ADCC	Antibody-Dependent Celular Citoxicity
AE	Adverse Events
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cells
APC-Cy7	AlloPhycoCyanin-Cy7 conjugate
APOBEC3	enzyme catalytic polypeptide like 3
ART	Antiretroviral Therapy
ARV	Antiretroviral
ATI	Antiretroviral treatment interruption
bNAbs	broadly Neutralizing Antibodies
BV	Brilliant Violet
CA-RNA	Cell-Associated RiboNucleic Acid
cART	combined AntiRetroviral Therapy
CCR5	C-C chemokine Receptor type 5
CCR7	C-C chemokine Receptor type 7
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CD14	Cluster of Differentiation 14
CD16	Cluster of Differentiation 16
CD19	Cluster of Differentiation 19
CD25	Cluster of Differentiation 25
CD28	Cluster of Differentiation 28
CD38	Cluster of Differentiation 38
CD45RA	Cluster of Differentiation 45 splice variant RA
CD49	Cluster of Differentiation 49
CD69	Cluster of Differentiation 69
CDC	Centers for Disease Control
CEF	Cytomegalovirus, Epstein-Barr Virus and Influenza Virus
ChAdV63	Chimpanzee Adenovirus 63
CMV	CytoMegaloVirus
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTL	$CD8^+$ cytotoxic T lymphocytes
CXCR4	C-X-C chemokine Receptor type 4
CYP3A4	Cytochrome P450 3A4
ddPCR	droplet digital Polymerase Chain Reaction
DNA	DeoxyriboNucleic acid
EC	Elite Controllers

ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunosorbent Spot
FBS	Fetal Bovine Serum
FDA	Food and Drug administration
FITC	Fluorescein isothiocyanate
FSC	Forward Side Scatter
GAG	Group-specific antigen
GALT	Gut-Associated Lymphoid Tissue
GMP	Good Manufacturing Practice
HAART	Highly Active Antiretroviral Treatment
HDACi	Histone DeACetylation inhibitors
HESN	Highly exposed seronegative individual
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
ICS	Intracellular Cytokine Staining
IFN	Interferon
IFN-γ	Interferon gamma
IL-2	InterLeukin-2
KIR	killer immunoglobulin-like receptors
LoC	Loss of HIV Control
LRA	Latency Reversing Agent
LTNP	Long-Term Non Progressors
LTR	Long Terminal Repeats
MAP	Monitored Anti-retroviral Pause
MIP.	Macrophage inflammatory protein
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIP.	Macrophage inflammatory protein
MOI	multiplicity of infection
MSA	multiple-sequence alignments
MVA	Modified Vaccinia Ankara
NK	Natural Killer cells
OLP	Overlapping Long Peptides
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-Buffered Saline
PCR	Polimerase Chain Reaction
PD-1	Programmed cell Dead Protein 1
PerCP	peridinin chlorophyll protein
PHA	PhytoHemAgglutinin

PI3K	phosphatidylinositol 3-kinase
PKC	Protein Kinase C
PrEP	Pre-exposure Prophylaxis
PTC	Post Treatment Controllers
pVL	plasma Viral Load
RMD	Romidepsin
RNA	RiboNucleic Acid
RMPI	Roswell Park Memorial Institute
RT	Reverse Transcriptase
RT-PCR	Transcription quantitative Polimerase Chain Reaction
SAE	Serious Adverse Event
SAMHD1	SAM domain and HD domain
SCA	Single Copy Assay
SSC	Side Scatter
SCT	Stem Cell Transplantation
SFC	Spot-Forming Cells
SIV	Simian Immunodeficiency Virus
STI	Structured Treatment Interruptions
TCR	T cell receptors
TLR	Toll-like Receptor
TNFα	Tumor Necrosis Factor alpha
VC	Viremic Controller
VIA	Viral Inhibition Assay
VISCONTI	Virological and Immunological Studies in CONtrollers after Treatment Interruption
VL	Viral Load
ZFNs	Zinc fingers nucleases

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ABSTRACT

Human Immunodeficiency Virus (HIV) infection causing the Acquired Immune Deficiency Syndrome (AIDS) is one of the major pandemics of our time. In 2018, 1.7 million of new infections occurred and over 37.9 million people were estimated to be living with HIV-1 infection (UNAIDS). Although combined antiretroviral therapy treatment (cART) can suppress viral replication and has led to dramatically reduced AIDS-related mortality, it is not able to cure the infection. Moreover, cART present some major inconvenients, including the need for lifelong adherence, daily dosing and several long-term side effects. In addition, the cost of cART is high, which makes it not accessible to many individuals living with HIV, especially among people in low- and middle-income countries. For these reasons, the development of an effective preventive vaccine and a cure strategy are of greatest importance to achieve the end of the HIV pandemic.

However, the development of a preventive vaccine and an effective cure for HIV has proven to be one of the major scientific challenges of our times. In order to develop a cure, we need to better understand the mechanisms that underpin immune-mediated control of HIV so that these insights can be incorporated into new immune interventions. Moreover, understanding the processes by which the virus is able to persist upon cART and how we can reverse HIV latency is critical in order to develop new interventions that aim to halt HIV persistence. We believe that a successful HIV cure intervention will require a combined approach able to both, reactivate the virus as well as to induce an immune response able to clear latently infected cells.

In this thesis, we aim to provide a better understanding of the mechanisms mediating HIV immune control as well as to define potential immune correlates of control in intervention clinical trials. The first two chapters of the thesis are focused on identifying potential virological and immune correlates of control that could be used as predictive markers when evaluating efficacy outcomes in intervention clinical trials.

The results presented in Chapter I support the evidence that the functionality of $CD8^+$ T cells is essential for the maintenance of natural HIV control. This leads us to propose the assessment of functional HIV-1 specific $CD8^+$ T cell responses using viral inhibition assays as a potential predictor of viral control when evaluating the immunogenicity of new immune intervention.

The study of virologic and immune markers associated with viral outcomes after a treatment interruption in the RISVAC03 therapeutic vaccine trial in Chapter II demonstrated that HIV-1 proviral DNA levels was associated with time to viral rebound. The proviral DNA levels were also predictive of the peak viral load reached after the treatment was interrupted. Importantly from a safety point of view, levels of proviral reservoirs did not increase after 2 weeks of treatment interruption even though half of

participants had detectable plasma viremia, suggesting that there is a window of time to prevent reservoir re-seeding.

The second part of the thesis is focused on the BCN02 clinical trial, the first kick&kick clinical trial that has shown some level of post-intervention viral control. The BCN02 clinical trial tested in early-treated individuals the effect of a therapeutic vaccine designed to induce HIV-specific responses towards the most conserved domains of HIV combined with the histone deacetylase inhibitor romidpesin, given as a latency reversing agent (LRA).

In Chaper III we evaluated the safety, immunogenicity and the effect on the viral reservoir of the combined intervention as well as the effects of the treatment on the viral rebound kinetics after a short, monitored antiretroviral pause. We detected high levels of vaccine-induced T cell responses and romidepsin-driven changes in histone acetylation and HIV transcription activity which was associated with a slight reduction of the viral reservoir.

In order to determine whether the LRA romidepsin had a detrimental effect on the vaccine-induced immune cells that could explain the limited impact on the viral reservoir observed in BCN02, Chapter IV of the thesis addressed the in vivo impact of romidepsin administrations on vaccine-induced T cells. We showed that despite transient increases in the frequency of apoptotic cells, activation of vaccine-induced T cells and a decrease in polyfunctionality phenotypes over romidepsin treatment, the antiviral capacity of $CD8^+$ T cells was not impaired. Altogether, the results from the second part of the thesis support the potential kick&kill strategies in achieving a durable immune-mediated HIV-1 control. Additionally, the comprehensive characterization of the *in vivo* effects of romidepsin could be incorporated into optimized combination regimens of LRA and vaccines in the future.

In summary, this work provides an overview of one of the many strategies that are being pursued to attain an HIV cure, starting from finding better correlates of control and finally testing an HIV cure strategy in human clinical trials. We believe that the key for an effective HIV cure relies on finding a way to reactive the virus from latently infected cells and finding an optimized vaccine candidate able to effectively re-educate the immune response to clear infected cells. Achieving the best design and combination of these two components has proved to be very challenging. We hope that all the scientific knowledge in the HIV field obtained during the last 30 years, along with all the currently ongoing and future clinicals trials testing new therapeutic strategies such as the one presented in this thesis, will ultimately contribute to the development of an effective HIV cure strategy.

RESUM

El virus de la immunodeficiència humana (VIH), causant de la síndrome de la immunodeficiència adquirida (SIDA), és una de les majors pandèmies del nostre temps. Al 2018, van haver-hi 1,7 milions noves infeccions i es calcula que actualment, més de 37,9 milions de persones viuen amb el VIH-1 a tot el món. Tot i que la teràpia antiretroviral combinada suprimeix la replicació del virus i ha reduït la taxa de mortalitat deguda a la SIDA i a altres malalties relaciones, no és una cura definitiva per la infecció del VIH. La teràpia antiretroviral no està exempta d'inconvenients, entre d'altres, la necessitat d'una alta adherència al tractament –el qual s'ha de diàriament- o la possible aparició d'efectes secundaris al llarg termini. A més, el seu alt cost fa que no sigui accessible per tots els individus que viuen amb el VIH, especialment per les persones que viuen en països en vies de desenvolupament. Així doncs, per poder acabar definitivament amb aquesta pandèmia és de gran importància desenvolupar una vacuna preventiva efectiva i una estratègia de cura.

Tanmateix, el desenvolupament d'una vacuna preventiva i una cura efectiva és una de les tasques més difícils del panorama científic actual. Per poder desenvolupar una cura, primer s'ha de tenir un millor coneixement dels mecanismes pels quals la immunitat de l'hoste pot controlar la infecció del VIH, i així poder incorporar aquests coneixements en noves intervencions terapèutiques. A més, és essencial entendre el procés pel qual el virus és capaç de persistir durant el tractament antiretroviral i com podem revertir el procés de latència del VIH. Una intervenció de cura pel VIH probablement haurà de combinar estratègies que puguin reactivar el virus i alhora, induir una resposta immunològica capaç d'eliminar les cèl·lules infectades pel virus que romanen en estat latent.

En aquesta tesis, els nostre objectiu ha estat millorar el coneixement sobre els mecanismes immunològics involucrats en el control del VIH, i definir potencials marcadors associats amb control de la infecció que es puguin usar quan l'eficàcia de d'assaigs clínics.

Els resultats del primer capítol aporten evidència de que la funcionalitat de les cèl·lules T $CD8^+$ VIH-1 específica és essencial per a mantenir el control de la infecció pel VIH al llarg del temps. D'aquesta manera, creiem que testar la funcionalitat de les respostes $CD8^+$ VIH-1 específiques utilitzant assaigs d'inhibició viral *in vitro* podria ser un possible predictor de control viral quan avaluem una nova teràpia.

En el capítol II, l'estudi realitzat abans i durant l'aturada de tractament antiretroviral en pacients que varen participar en l'assaig clínic de vacuna RISVAC03 demostra que el nivell de DNA proviral del VIH s'associa amb el temps a produir-se el rebot viral un cop s'atura el tractament. En el mateix capítol es mostra que els nivells de DNA proviral del virus també són predictius del pic de virèmia que s'assoleix després de l'aturada de

tractament. A més a més, es va observar que els nivells de reservori viral no van augmentar després de dues setmanes d'aturada de tractament, tot i que la meitat de pacients ja tenien càrrega viral detectable en plasma, suggerint que hi ha una finestra de temps per evitar l'augment del reservori viral durant les aturades de tractament.

La segona part de la tesi es centra en l'assaig clínic BCN02, el primer assaig "kick&kill" que ha demostrat un cert nivell de control virològic després d'una intervenció immunoterapèutica. L'estudi BCN02 consistia en testar, en individus que varen iniciar el tractament antiretroviral en la fase aguda de la infecció (els primers mesos després de l'adquisició del VIH), una vacuna terapèutica dissenyada per generar respostes VIH especifiques contra les regions més conservades del virus, juntament amb l'inhibidor d'histona deacetilasa romidepsina, que s'utilitza com a agent reactivador de la latència.

Al capítol III hem avaluat la seguretat, la immunogenicitat i l'efecte en el reservori viral d'aquesta intervenció combinada. També s'ha analitzat l'eficàcia de la intervenció en les cinètiques de rebot viral durant una pausa breu del tractament antiretroviral. En l'estudi BCN02 es van detectar alts nivells de respostes de cèl·lules T induïts per la vacuna, juntament amb canvis en l'acetilació de les histones i transcripció del VIH durant l'administració de romidepsina, que s'han associat amb una reducció petita del reservori viral.

Per determinar si el poc impacte en el reservori viral va ser degut a una poca activitat de la romidepsina o per un possible efecte tòxic del fàrmac sobre les cèl.lules estimulades per la vacuna, es va realitzar el treball presentat en el capítol IV de la tesi. Els nostres resultats mostren que tot i que la romidepsina augmenta la freqüència de cèl·lules apoptòtiques i cèl·lules activades per la vacuna i en disminueix la seva polifunctionalitat, la capacitat antiviral de les cèl·lules $CD8^{+}$ no es veu veure afectada. La caracterització realitzada de l'efecte de la romidepsina en aquesta intervenció s'hauria de tenir en compte alhora d'optimitzar els règims i les dosis de noves estratègies de cura del VIH que combinin l'ús de reactivadors de la latència amb vacunes terapèutiques.

En resum, aquest treball ha estudiat en profunditat vàries de les diverses estratègies que s'estan testant per aconseguir una cura per la infecció pel VIH, començant per la recerca de millors marcadors de control en infecció natural, l'ús de vacunes terapèutiques i la seva combinació amb reactivadors de la latència. Creiem que la clau per a aconseguir una cura efectiva contra el VIH rau en trobar una manera per reactivar el virus de les cèl·lules latentment infectades i trobar un candidat de vacuna òptim que reeduqui de manera eficaç el sistema immunitari per a que sigui capaç d'eliminar les cèl·lules infectades. Aconseguir el disseny i la combinació d'aquests dos components és un gran repte que només es podrà aconseguir tenint en compte tot el coneixement generat en els últims 30 anys, juntament amb els resultats dels nous assaigs clínics que s'estan testant, inclosos els presentats en aquesta tesi.

INTRODUCTION

1. The *human immunodeficiency virus* (HIV)

1.1 The origin of the HIV pandemic and its epidemiology

The first cases of acquired immune deficiency syndrome (AIDS) were reported in 1981, particularly in California, among individuals who suffered from Kaposi's sarcoma and showed susceptibility to *Pneumocystis carinii* pneumonia, previously described in immunocompromised patients [1]. Two years later, in 1983, the human immunodeficiency virus (HIV)-1 was described by two independent groups led by Luc Montaigner along with Françoise Barré-Sinoussi and Robert Gallo and his team [2,3]. In 1985, a T cell tropic virus was discovered in monkeys, and it was shown to be related to HIV [4]. From that point on, many studies have investigated the similarities and differences between simian immunodeficiency virus (SIV) and HIV-1 infection, leading to the conclusion that the origin of the HIV-1 pandemic was a zoonotic transfer from chimpanzees to humans [5,6]. HIV-2 was discovered in 1986, being related to HIV-1 but being less virulent and mainly found in West Africa [7,8]. Its emergence was also originated by a zoonotic transfer from sooty mangabeys to humans [9,10].

Since the pandemic started, 76.1 million people have been infected by HIV and 35 million people have died due to AIDS or related diseases. To date, 36.7 millions of people in the world are infected by HIV, of which 25.7 live in Africa [11]. Although reported infections decrease over the years, especially in developed countries, yet in 2017 there were 1.8 million new infections. Actually, every day 5.000 people get infected around the world. Despite the fact that there are 19.5 million people under antiretroviral therapy, HIV still causes 1 million deaths per year.

1.2 HIV: classification and structure

HIV is a member of the retroviridae family of viruses, part of the lentivirus family [12], which are enveloped viruses with a single-stranded positive-sense RNA [13]. HIV is classified in two subtypes: HIV-1 and HIV-2. HIV-1 is the major cause of the AIDS pandemic and it is spread globally, while HIV-2 is less virulen and affects fewer and affects fewer people. In this thesis we have mainly focused on HIV-1 infection, so we will describe in greater detail its structure.

HIV-1 is a spherical particle of 80-150nm containing two identical strains of RNA covered by a conic capsid formed by the viral protein p24, which also includes the viral proteins needed for viral replication (Figure 1). The capsid is surrounded by a

phospholipid bilayer envelope stemming from the host membrane which contains the viral envelope glycoproteins encoded by the virus [14].

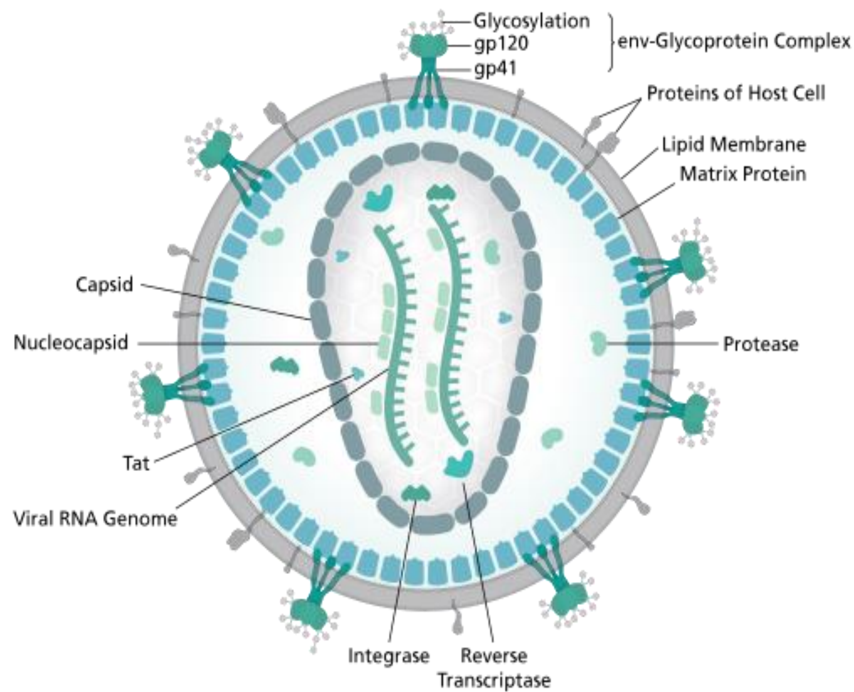

Figure 1: HIV-1 virion structure. Membrane of the viral enveloped is formed by host cell membrane with proteins gp41 and gp120 inserted into the lipid membrane. Inside the envelope, p17 gag matrix surround the conical-shaped capsid formed by p24 protein, which contains the two strains of viral RNA associated with other enzymes including reverse transcriptase, integrase and protease. Image by Thomas Splettstoesser (www.scistyle.com).

The HIV-1 genome is approximately 10 kilobases (kb) in size and contains nine genes that encode for sixteen viral proteins (Figure 2) [15–17]. The group-specific antigen (gag) sequence encodes for structural proteins: the matrix protein p17, the capsid protein p24, the nucleocapsid p7 and the late assembly protein p6. The polymerase (pol) gene codes for viral enzymes required for the virus life cycle: the protease (Prot), the reverse transcriptase (RT) which has two major subunits, p66 and p51. The envelope gene (env) codes for the precursor glycoprotein (Gp)160 that contains Gp120 and Gp41 subunits, which form trimer spikes (3 Gp120 subunits along with 3 Gp41 subunits) that are exposed on the virion membrane. When the virus adheres to target cells, the spikes allow for the fusion of the virion membrane with the target cell membrane favoring the entry of the viral genome into the target cell. Other lentiviral genes are the transcriptional activator (Tat) and the regulator of viral gene expression (Rev), which are regulatory proteins: Tat is required for the elongation of viral transcripts, while Rev promotes nuclear export of spliced viral RNAs. Viral protein U (Vpu) or viral protein X (Vpx) in the case of HIV-2, viral

infectivity factor (Vif) and negative effector (Nef) are considered accessory proteins that regulate viral replication and can mediate evasion of intrinsic host defense mechanisms.

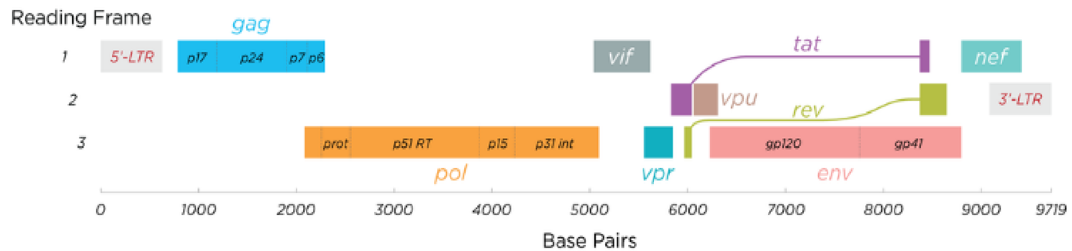

Figure 2: HIV-1 genome. The HIV genome has long terminal repeats (LTRs) at the genome extremes which allows the virus to insert its genetic material into host genome. The genes (gag, pol, vif, vpr, tat, rev, vpu, env and nef) are pointed out with different colors. The coding sequences of rev and tat require RNA splicing since they are separated in the genome. Image by Thomas Splettstoesser (www.scistyle.com).

1.3 The HIV-1 life cycle

The life cycle of HIV-1 (Figure 3) begins when the envelope glycoprotein Gp120 binds to the host cell surface receptor CD4 [18] in addition to one of the chemokine co-receptors C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) of the target cell [19]. As soon as the interaction occurs, the HIV-1 envelope protein changes its conformation, exposing the repetitive coiled coils of Gp41 [20], which allow the fusion of HIV-1 with the host cell membrane. Hence, the viral core with the viral genome and other HIV-1 proteins enter the host cell [21]. The shell of the capsid disintegrates, and the viral RNA is released into the cytoplasm. Viral DNA is formed by reverse transcription using the everse ranscriptase enzyme [22]. Due to the lack of fidelity of the RT and its ability to switch between RNA templates [23,24], the retrotranscription of the DNA generates many genetic variants of the single strain RNA molecule. The conversion of RNA to double stranded DNA allows the formation of the pre-integration complex, which is helped by Vpr, to enter into the nucleus of the target cell [2]. There, the HIV-1 integrase protein integrates HIV-1 DNA into the cell's host DNA, often in "hot spots" of increased transcriptional activity [25]. The host's normal transcription machinery then transcribes HIV-1 DNA into multiple copies of new HIV-1 RNA. This process takes place when the cell is activated [26], otherwise, the virus remains in a latency state forming the HIV-1 reservoir [27]. If HIV-1 DNA is transcribed, translation takes place in the cytoplasm and results in the production of Tat, Rev and Nef proteins. Tat migrates to the nucleus

in order to increase the production of full-length mRNA transcripts [28], that will be bound and transported to the cytoplasm by Rev [29]. As Gag, Pol and Env gp160 are being translated, HIV-1 RNA along with the before-mentioned proteins assemble into immature HIV-1 particles. These particles move to the surface and bud from the host cell membrane, activating the protease enzyme that cleaves newly synthesized Gag and Gag-Pol proteins. Cleavage results in formation of the mature conical capsid core, packaging RNA and HIV accessory proteins, that now forms an infectious virion. New virions are estimated to be viable for less than 8 hours in plasma if they do not enter a target host cell [30]. As a matter of fact, mathematical models have shown that HIV-1 takes 1.2 days to replicate and that infected cells survive for an average of 2.2 days [30]. Despite this short survival time, during acute infection, total virion production is around 6.8 million viral particles per day [31].

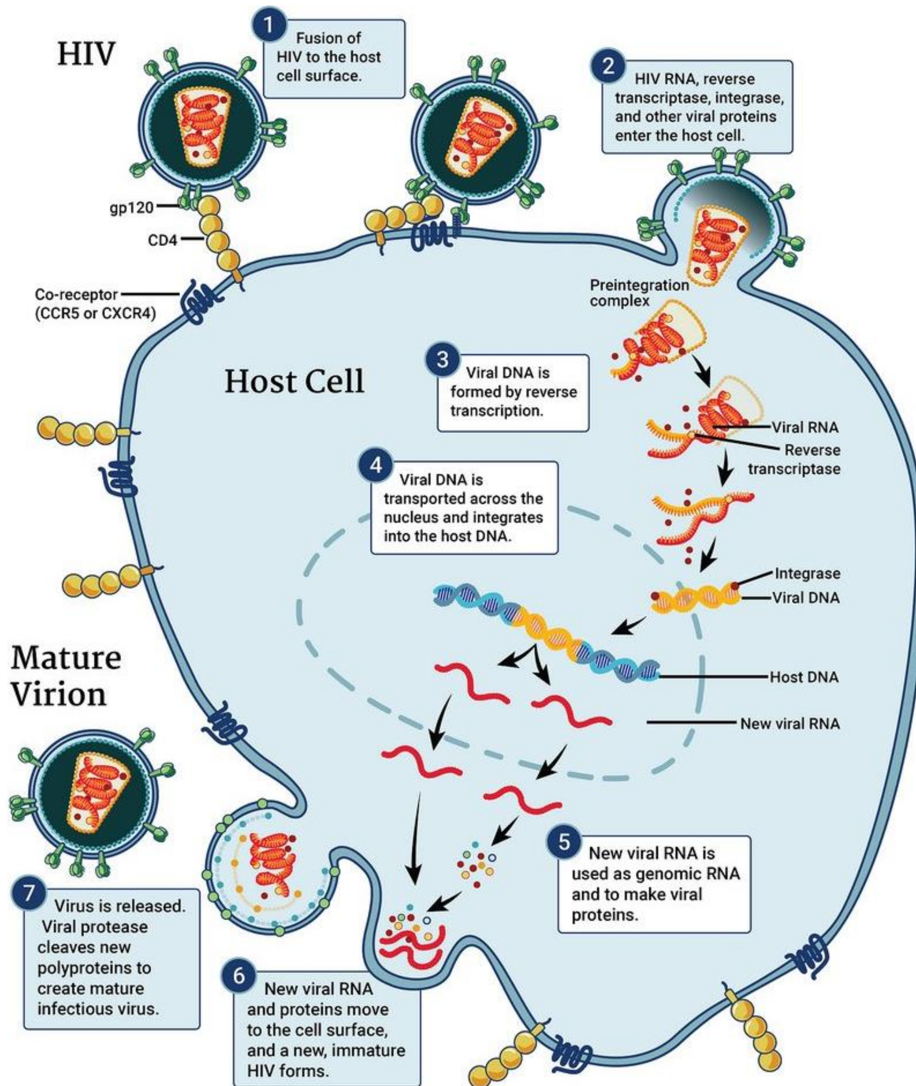

Figure 3: Overview of HIV-1 replication cycle. The different stages of the HIV life cycle are binding, fusion, reverse transcription, integration, replication, assembly and budding. Image from the NIAID Flickr site.

1.4 Pathogenesis of HIV-1

HIV-1 infection course begins with acute infection (generally asymptomatic or causing an influenza-like illness 2-4 weeks after exposure) advances to a chronic stage and in absence of treatment, finally progresses to AIDS (Figure 4). Infection of HIV-1 most often results from the successful transmission and subsequent propagation of a single virus variant, called transmitted or founder virus [32]. This is due to a bottleneck effect that results in an establishment of productive infection from a single HIV-1 variant in 80% of transmissions [33]. However, shortly after transmission, HIV-1 evolves to multiple genetic variants that fuel the productive clinical infection. Early infection is characterized by infection of activated $CD4^+$ T cells in mucosal gut-associated lymphoid tissue (GALT). HIV-1 fuses via one of the two chemokine receptors, mostly to CCR5, expressed on macrophages and some memory T cells, during new infections [33]. Over the course of the infection, the virus usually evolves to CXCR4-tropism. Since CXCR4 is highly expressed in distinct T cells, the change of tropism has been related to late stages of the disease and virulence [34]. HIV-1 infection causes a depletion of activated and memory $CD4^+$ T cells resulting in alterations of the GALT that facilitate microbiome invasion, and immune activation with high production of pro-inflammatory cytokines and T cell proliferation [35–37]. At that point, dendritic cells can also capture the virus and migrate to secondary lymphoid tissues, where the virus replicates and starts disseminating [38,39]. The spread of viral particles leads to detectable viremia in blood that can reach 10,000,000 copies/milliliter within 2-4 weeks after the initial infection. In this phase, individuals may develop a virus-like illness, known as the acute retroviral syndrome, characterized by headache, retro-orbital pain, muscle aches, sore throat, fever, swollen lymph nodes and a non-pruritic macular erythematous rash that can last from days to weeks. While the virus disseminates by infecting $CD4^+$ T cells and to a leaser extent macrophages, it can also spread via dissemination of viral particles stored in dendritic cells, the adaptive host immune system generates both cell-mediated and humoral response [40,41]. The immune response generated is able to decrease levels of viremia although is not able to eliminate the infection [42].

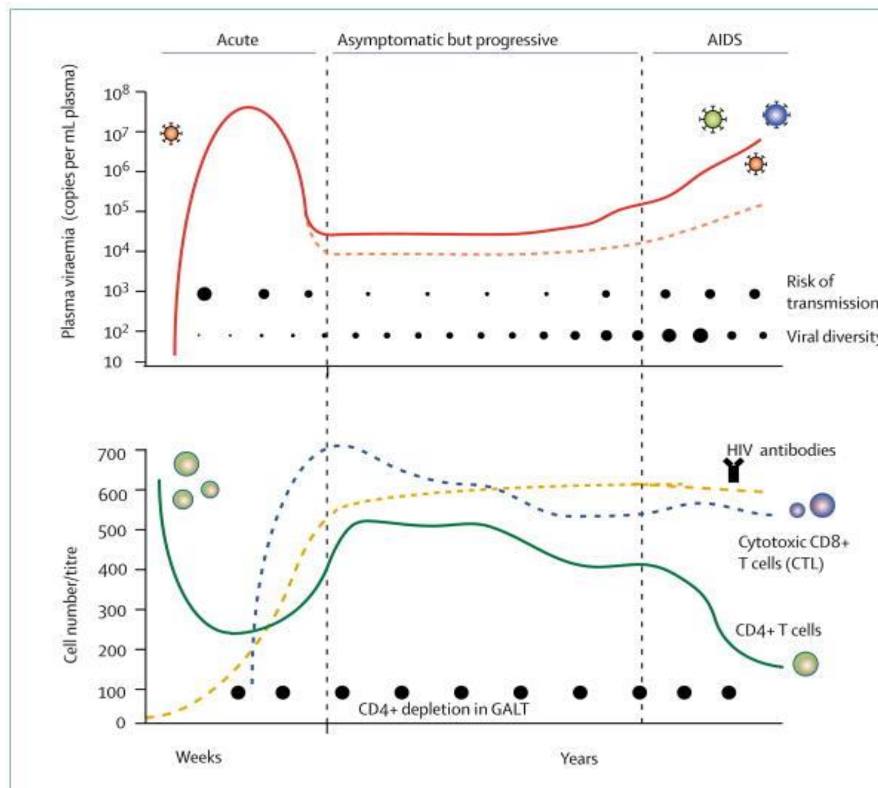

Figure 4: The course of HIV-1 infection. On the upper figure, evolution of the plasma viremia is shown over the evolution of HIV-1 (red and orange doted lines represent the difference of viral set-points among individuals). At the bottom figure, evolution of the $CD4^{+}$T cells dynamics (green), presence of cytotoxic $CD8^{+}$ T cells (blue) and specific antibodies (yellow) are shown during the evolution of the disease. Image from Simon et al, Lancet 2006 [43].

The analysis of samples from individuals identified and tested early after infection with HIV-1 allowed for the classification of the first weeks following infection into clinical stages defined by the sequential emergence of assay reactivity [44]. Figure 5 shows the classification of primary HIV-1 infection, depending on the results of the assays represented. The time between infection and the first detection of viral RNA in the plasma is referred to as the eclipse phase. Plasma virus levels then increase exponentially, peaking at 21–28 days after infection, and this is followed by a slower decrease in plasma viral RNA levels. Patients can be categorized into Fiebig stages I–VI, which are based on a sequential gain in positive HIV-1 clinical diagnostic assays (viral RNA measured by PCR, p24 and p31 viral antigens measured by enzyme-linked immunosorbent assay (ELISA), HIV-1-specific antibody detected by ELISA and HIV-1-specific antibodies detected by western blot. Patients progress from acute infection through to the early chronic stage of infection at the end of Fiebig stage V, between 13 and 45 days following infection, as the plasma viral load begins to plateau [44].

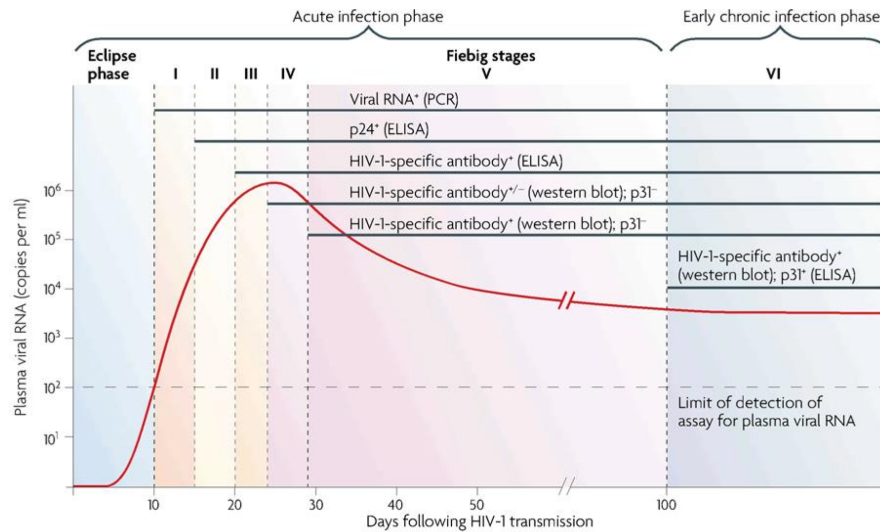

Figure 5: Representation of the states in the of acute HIV-1 infection defined by Fiebig. Image from Mc Michael Nat Rev Immunology 2010 [45].

The chronic stage is the next phase of the disease, which is generally asymptomatic for many years and the viremia is stabilized at an individual set-point. However, there is persistent viral replication and the number of $CD4^+$ T cell counts decreases gradually [46]. The course of the infection progresses to the final phase, called AIDS phase, in which the balance between the viral replication and the immune response is lost, resulting in an increase of the viral replication and a steep loss of $CD4^+$ T cells. The damage on lymphocytes makes individuals more susceptible to opportunistic infections that can cause organ failure, cachexia, degeneration of the central nervous system and tumors, what defines the AIDS stage of the disease. AIDS-defining diseases by the Center of Disease Control include Kaposi's sarcoma, Lymphoma, Burkitt's or Pneumonia among others.

Of note, some HIV-1 infected individuals have been identified that remain clinically stable and do not present any AIDS-defining condition for decades after HIV-1 acquisition. These individuals have been referred as long-term non-progressors (LTNP) and are able to control viral replication to low levels (plasma RNA levels lower than 2,000 copies/mL) and maintain normal $CD4^+$ T cell counts over time. Few LTNP are considered elite controllers (EC), who maintained undetectable viral loads for extended periods of time.

1.5 Immunity in natural HIV-1 infection

1.5.1 Innate response

HIV-1 infection leads to an induction of innate, cell-mediated and humoral responses. The first line of defense against HIV-1 infection is the innate immunity based on epithelial barriers, the complement system and antigen presenting cells with phagocytosis capacities [47]. HIV-1 is detected by pattern recognition receptors (PRR) such as toll-like-receptors (TLR) [48] and retinoid acid-inducible gene (RIG)-like receptors (RLR) [49]. Although the interaction between HIV-1 and the innate system is not well established and is widely different depending on cell types and tissues involved, it is clear that the presence of HIV-1 induces activation of immune cells including plasmacytoid dendritic cells (pDC) and natural killer cells (NK) [47]. NK cells are the essential antiviral effector cells of innate immune responses [50], being able to provide a rapid immune defense by triggering cytokine release, causing apoptosis and lysis of infected cells [51]. NK activation depends on the balance between the interaction of activating and inhibitory receptors [52]. NK cells can recognize virally infected cells through their various receptors, shown in Table 1. Functional studies suggested that NK cells can mediate immune pressure to HIV-1 by HLA/KIR interactions [52].

Receptor family	Ligands	Activating/ Inhibitory
Killer-immunoglobulin like receptor (KIR)	HLA-A, HLA-B, HLA-C, HLA-G	Activating and Inhibitory
Natural-killer, Group-2 member D (NKG2D)	MIC-A, MIC-B, RAE-1, ULBP1, ULBP2, ULBP3, ULBP4	Activating
CD94/NKG2 members A/C/E	HLA-E	Activating and Inhibitory
Natural cytotoxicity receptors (NCR)	Viral ligands	Activating
CD16/ FCγ receptor III	IgG	Activating
Ly49	MHC Class I	Activating and Inhibitory

Table 1: NK cells receptor. Human NK activating and inhibitory receptors are listed. Adapted from Pegram H et al 2011 [52].

NK cells also play a role in the adaptive immune response mediating antibody-dependent cellular cytotoxicity (ADCC). FCγ receptors III (CD16) on NK cells bind

to IgG antibodies inducing cellular activation and target cell lysis. This ADCC activity has been linked to slower HIV-1 disease progression in elite controllers [53] and, more recently, has been shown to play an important role in developing protective vaccine-elicited IgG responses against HIV-1 [54]. With the increasing interest in ADCC activity related to HIV-1 control, several studies are characterizing antibodies that are able to trigger this type of activity [55,56].

1.5.2 Host restriction factors

During HIV-1 replication in the target cell, the virus interacts with several proteins of the host cell, which can help viral replication, or on the contrary, suppress it. These host cellular factors that inhibit HIV-1 replication are termed host restriction factors and play an important role in the initial stages of the viral life cycle. Some of the best studied host restriction factors include: poliprotein B mRNA editing enzyme catalytic polypeptide like 3 (APOBEC3), tripartite motif 5α (TRIM5α), SAM domain and HD domain 1 (SAMHD1) and tetherin. However, over time HIV-1 has evolved developing strategies to evade these host restriction factors by the action of its accessory proteins, especially Vpu and Vif for HIV-1 and Vpx for HIV-2. Mechanisms of action of host restriction factors and HIV-1 proteins involved on the evasion of those mechanisms are shown in Table 2.

Restriction Factor	Mechanism of action	HIV protein involved on the evasion
APOBEC3	Interferes with viral replication by hypermutation on the cDNA	Vif binds to APOBEC3 and recruits it for ubiquitination and degradation [57]
TRIM5α	Accelerates capsid fragmentation and degradation of viral reverse transcriptase complex [58]	Not known
SAMHD1	Prevents synthesis of viral DNA by reverse transcription [59]	Vpx (in HIV-2): antagonizes SAMHD1 activity
tetherin	Prevents virion budding in certain cell types by binding to viral envelope	Vpu neutralizes tetherin [60]

Table 2: Best example of host HIV restriction factors. Host restriction factors and their mechanism of action against HIV infection are listed, as well as the HIV proteins that participate in the evasion of such mechanisms.

1.5.3 T cell response

Adaptive immune responses to HIV-1 consist in both HIV-specific T cell and humoral immune response. Within 3 weeks after infection, an expansion of HIV-specific $CD8^+$ cytotoxic T lymphocytes (CTL) can be observed, associated with the initial decay of the viremia after reaching its peak around day 13 [61,62]. The importance of CTL responses was already described 20 years ago, with studies in acute infection showing a temporal correlation between early HIV-specific CTL responses and viral control [61,63]. This was supported by studies in SIV macaque models where $CD8^+$ T cell depletion was associated with an increase in viremia, while restoring the CD8 T cell population resulted in reestablishment of controlled SIV replication [64]. Moreover, particular virus-specific T cell responses restricted by human leukocyte class I antigens (HLA) were associated with relative slow disease progression [65]. In addition, highly exposed seronegative individuals (HESN) presented HIV-specific CTLs responses that have been described to mediate protection from HIV-1 infection [66,67]. Further evidence for the importance of CTL responses during HIV-1 infection is the evident evolution of the virus under CTL-mediated immune selection pressure [68]. It has been well described that HIV-specific responses are different between individuals based on their HLA restriction since each HLA is able to present certain HIV-1 epitopes [69]. The fact that the virus escapes from CTL responses, even if it results in a decreased HIV-1 replicative capacity, suggests that CTL responses directly affect the viral replication [70,71].

The HLA nomenclature refers to the protein products encoded in the major histocompatibility complex (MCH) in humans. The MHC genes are located on chromosome 6 and encode for transmembrane highly polymorphic proteins, whose function is to present antigens to T lymphocytes. HLA molecules are grouped into classical HLA class I (A, B and C), HLA class II and class III [72]. While MHC class I molecules present antigenic peptides derived from proteins synthesized inside the cell, MHC class II molecules present peptides derived from proteins in endosomal compartments of the cell [73,74]. MHC class III encodes for components of the complement system.

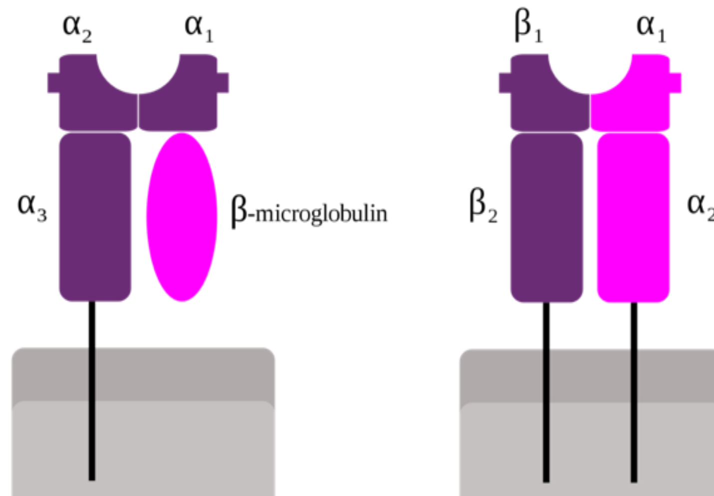

Figure 6: Schematic representation of MHC class I molecule (left) and MHC class II molecule (right). The MHC class I heavy chain consists of three α-domains and one β2-microglobulin unit while MHC class II molecule consists of an α-chain and a β-chain, each with like domains. The peptide-binding groove is situated between domains α1 and α2 in class I and between β1 and α1 in class II. Image from atropos.

As shown in Figure 6, class I and class II molecules share an overall similar structure. The peptide-binding groove is composed by two domains, α1 and α2 for MHC class I molecules and β1 and α1 in MHC class II molecules. The groove in MHC class I molecules is closed at both ends by conserved tyrosine residues and leading to a restriction of peptides' length from to mostly 8–10 residues and with anchor residues at position P2 or P5/6 and PΩ (residue at C-terminal) that fit into the epitope binding pockets [75–77]. However, longer epitopes have been eluted as well [78]. In contrast, peptides presented in MCH class II are often longer, of 13-24 residues, as the antigen-binding groove of the class II molecules is open at both ends. The anchor residues in MHC class II molecules are at positions P1, P4, P6, and P9 [79,80]. Representation of binding grooves of MCH class I and II with a peptide is shown in Figure 7.

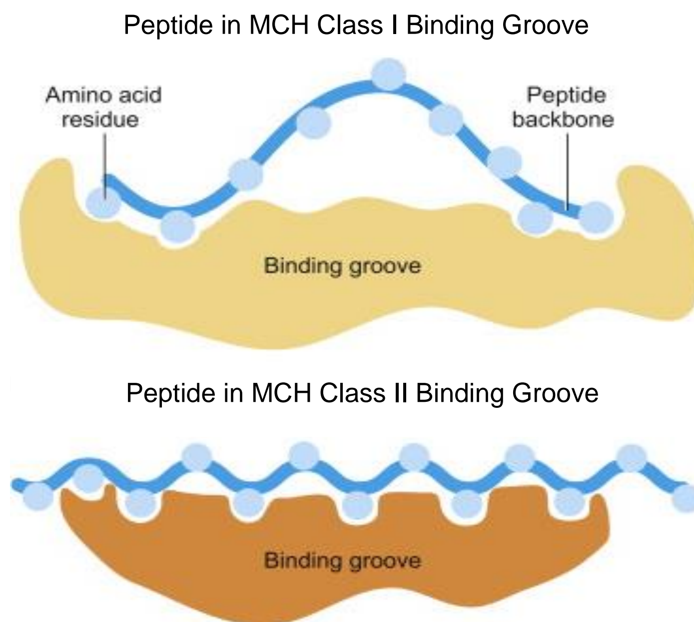

Figure 7: MHC class I and II molecules interacting with an epitope. Image adapted from "Primer to the Immune Response", 2014 [81].

The MHC loci is one of the most variable genetic loci in humans, with currently 17,099 alleles described for HLA Class I and 6,716 for HLA Class II (IPD-IMGT/HLA statistics available at www.ebi.ac.uk/imgt/hla, updated in July 2019). The HLA alleles nomenclature establishes that each HLA allele has a unique number corresponding to up to four sets of digits separated by colons. The length of the allele designation is dependent on the sequence of the allele and that of its nearest relative. All alleles receive at least a four-digit name, longer designations are only assigned when indicated (to indicate nucleotide substitutions, expression status, etc). Figure 8 shows an example of nomenclature factors of the HLA system.

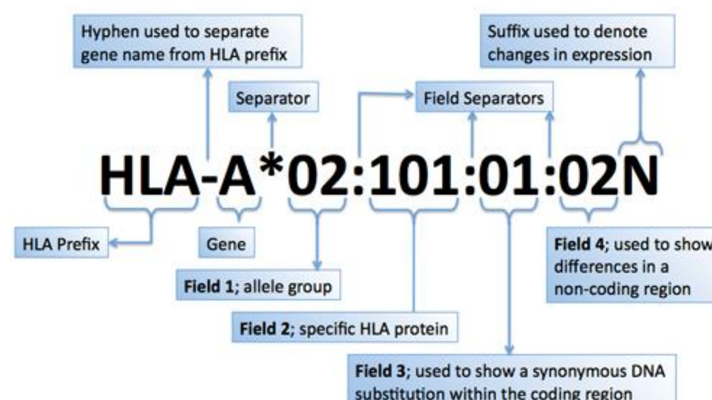

Figure 8: Schematic representation of nomenclature factors of the HLA system. Image from http://hla.alleles.org/

The diversity of HLA in the human population is one of the best strategies of immune defense on a population level, as from the variety of HLA derives the heterogeneity of T cell responses towards external pathogens. As mentioned, MHC class I presents peptides generally derived from proteins synthesized in the cell. Therefore, virally infected cells present antigenic proteins that have been degraded and translocated into the endoplasmatic reticulum where peptides are associated with the MHC class I molecules. Peptide:MHC complexes are then transported to the cell surface via the Golgi complex, where they can be recognized by $CD8^+$ T cells through T cell receptors (TCR) expressed on their membrane [82]. When $CD8^+$ T cells bind to their specific antigenic peptide:MHC complex, the TCR and other co-receptors cluster at the site of cell-to-cell contact, forming an immunological synapse. In the case of an anti-viral response, the immunological synapse leads to a polarization of the effector cell to orientate the release of effector molecules at the site of contact with the infected target cell. The differentiation of $CD8^+$ T cells into cytotoxic effector T cells results into release of cytotoxic molecules such as pore-forming perforin that induces cell lysis and granzyme that induces apoptosis of the infected cells. Most $CD8^+$ T cells also release cytokines that induce cytolysis such as INFγ and TNFα. INFγ induces an augmented expression of MHC class I molecules in infected cells, and activates other immune cells types such as macrophages, recruiting them towards the site of infection and enhancing recognition by other effector cells. On the other hand, extracellular antigens are taken up by APC by distinct endocytosis mechanisms and are degraded in endosomal compartments. This leads to antigen presentation on MHC class II molecules, which are recognized by $CD4^+$ T cells, triggering T cell activation, clonal expansion by cell division, differentiation into effector cells and activation of B cells into B cell blasts able to produce antibodies [83].

Compared to other viral infections such as hepatitis C virus (HCV) and hepatitis B virus (HBV), HIV-1 generates a very strong CTL immune response, specially directed towards Gag and Nef [84]. Therefore, the study of the CTL specificities towards HIV-1 has allowed for a very detailed characterization of class I CTL epitopes and the identification of so-called “optimal” CTL epitopes. Optimal CTL epitopes are defined as the shortest peptide that results in the highest stimulation of CTL response at the lowest peptide concentration. A list of these optimal epitopes of HIV-1 is published at Los Alamos HIV Immunology database and includes more than 300 well-characterized HIV-1 CTL epitopes along the HIV-1

proteome [85]. HIV-1 epitopes can be recognized by more than one HLA class I, with some of them being restricted by multiple different HLA class I alleles. Actually, HLA molecules can be clustered in groups of molecules that bind largely overlapping peptide repertoires. Different groups, denominated HLA supertypes, for HLA-A (6) and for HLA-B (6) alleles have been defined by their anchor specificity [86] (Figure 9). This classification improves the understanding of CTL promiscuity and is critical for epitope identification studies, for the analysis of immune driven viral evolution and for the rational design of vaccines aiming to provide a broad HLA coverage.

HLA A-supertypes											
A01											
A*0101	A*0103	A*0112	A*2609	A*2618	A*3012	A*3602		A*2501	A*3603		
A*2601	A*0104	A*0114	A*2610	A*2619	A*3202	A*3604		A*2502	A*7410		
A*2602	A*0106	A*0115	A*2611	A*2621	A*3205			A*2504	A*8001		
A*2603	A*0107	A*2604	A*2612	A*2623	A*3206			A*2622			
A*3002	A*0108	A*2605	A*2613	A*2624	A*3207			A*3110			
A*3003	A*0109	A*2606	A*2614	A*2626	A*3209			A*3203			
A*3004	A*0110	A*2607	A*2615	A*3006	A*3210			A*3204			
A*3201	A*0111	A*2608	A*2617	A*3009	A*3601			A*3208			
A01 A03											
A*3001	A*3008	A*3011	A*3014	A*3015				A*0252	A*3013	A*6806	A*6807
A01 A24											
A*2902	A*2901	A*2905	A*2909	A*2911				A*2913			
	A*2903	A*2906	A*2910	A*2912							
A02											
A*0201	A*0209	A*0224	A*0240	A*0257	A*0271	A*6827		A*0241			
A*0202	A*0211	A*0225	A*0243	A*0258	A*0272	A*6828		A*0242			
A*0203	A*0212	A*0226	A*0244	A*0259	A*0274			A*0250			
A*0204	A*0213	A*0227	A*0245	A*0261	A*0275			A*0260			
A*0205	A*0215	A*0228	A*0246	A*0262	A*0277			A*0273			
A*0206	A*0216	A*0230	A*0247	A*0263	A*0278			A*0284			
A*0207	A*0218	A*0231	A*0248	A*0266	A*0279			A*6815			
A*0214	A*0219	A*0236	A*0249	A*0267	A*0282						
A*0217	A*0220	A*0237	A*0251	A*0268	A*0283						
A*6802	A*0221	A*0238	A*0254	A*0269	A*0285						
A*6901	A*0222	A*0239	A*0256	A*0270	A*0286						
A03											
A*0301	A*0302	A*0316	A*1112	A*3105	A*3404	A*6812	A*7402	A*0265			
A*1101	A*0304	A*0317	A*1113	A*3106	A*3406	A*6813	A*7403	A*0280			
A*3101	A*0305	A*1102	A*1114	A*3109	A*6602	A*6814	A*7404	A*0309			
A*3301	A*0306	A*1103	A*1115	A*3111	A*6603	A*6816	A*7405	A*1106			
A*3303	A*0307	A*1104	A*1116	A*3304	A*6604	A*6819	A*7407	A*1122			
A*6601	A*0308	A*1105	A*1120	A*3305	A*6803	A*6821	A*7408	A*3112			
A*6801	A*0310	A*1107	A*1121	A*3306	A*6804	A*6822	A*7409	A*6805			
A*7401	A*0312	A*1108	A*1123	A*3307	A*6808	A*6824	A*7411	A*6820			
	A*0313	A*1109	A*3103	A*3402	A*6809	A*6825		A*6823			
	A*0314	A*1110	A*3104	A*3403	A*6810	A*6826		A*7406			
A24											
A*2301	A*2302	A*2310	A*2410	A*2422	A*2433	A*2440		A*2305	A*2442		
A*2402	A*2303	A*2403	A*2411	A*2423	A*2434	A*2443		A*2312	A*2444		
	A*2304	A*2405	A*2413	A*2426	A*2435	A*2446		A*2417	A*2452		
	A*2306	A*2406	A*2418	A*2427	A*2437	A*2447		A*2425			
	A*2307	A*2408	A*2420	A*2428	A*2438	A*2448		A*2430			
	A*2308	A*2409	A*2421	A*2429	A*2439	A*2449		A*2441			
Unclassified											
A*0102	A*0233	A*0276	A*1118	A*2414	A*2432	A*2616	A*3007	A*3308			
A*0113	A*0234	A*0281	A*1119	A*2415	A*2450	A*2620	A*3010	A*3401			
A*0208	A*0235	A*0315	A*2309	A*2419	A*2451	A*2904	A*3102	A*3405			
A*0210	A*0255	A*1111	A*2404	A*2424	A*2453	A*2907	A*3107	A*4301			
A*0229	A*0264	A*1117	A*2407	A*2431	A*2503	A*2914	A*3108	A*6817			

HLA B-supertypes												
B07												
B*0702	B*0704	B*0741	B*3540	B*5109	B*5134	B*5510		B*0707	B*3518	B*5112	B*8102	
B*0703	B*0706	B*0742	B*3541	B*5110	B*5135	B*5515		B*0709	B*3529	B*5113		
B*0705	B*0715	B*0743	B*3542	B*5111	B*5136	B*5517		B*0712	B*3530	B*5114		
B*1508	B*0719	B*3507	B*3543	B*5116	B*5138	B*5519		B*0714	B*3534	B*5120		
B*3501	B*0720	B*3508	B*3544	B*5117	B*5302	B*5603		B*0716	B*3537	B*5137		
B*3503	B*0721	B*3511	B*3545	B*5118	B*5306	B*5605		B*0717	B*3539	B*5304		
B*4201	B*0722	B*3514	B*3546	B*5119	B*5308	B*5613		B*0718	B*3551	B*5508		
B*5101	B*0724	B*3515	B*3554	B*5121	B*5310	B*5615		B*0723	B*3553	B*5511		
B*5102	B*0725	B*3521	B*3555	B*5123	B*5403	B*5616		B*0736	B*3558	B*5513		
B*5103	B*0726	B*3522	B*3557	B*5124	B*5404	B*7802		B*0737	B*3560	B*5514		
B*5301	B*0730	B*3524	B*3561	B*5126	B*5406	B*7804		B*3502	B*3806	B*5602		
B*5401	B*0731	B*3531	B*3910	B*5128	B*5407			B*3504	B*3807	B*5604		
B*5501	B*0733	B*3532	B*3916	B*5129	B*5503			B*3505	B*3917	B*5609		
B*5502	B*0734	B*3533	B*4204	B*5130	B*5504			B*3506	B*4206	B*5610		
B*5601	B*0735	B*3535	B*4205	B*5131	B*5505			B*3509	B*4406	B*5611		
B*6701	B*0739	B*3536	B*5105	B*5132	B*5507			B*3512	B*5104	B*5612		
B*7801	B*0740	B*3538	B*5108	B*5133	B*5509			B*3517	B*5106	B*8101		
B08												
B*0801	B*0807	B*0811	B*0815	B*0819	B*0821	B*0823	B*0825	B*0803	B*0812			
B*0802	B*0809	B*0813	B*0818	B*0820	B*0822	B*0824		B*0808	B*0816			
B27												
B*1402	B*1401	B*1593	B*3926					B*1405	B*3913			B*5518
B*1503	B*1403	B*1598	B*3927					B*1523	B*3915			
B*1509	B*1406	B*1599	B*3929					B*1568	B*3924			
B*1510	B*1407	B*2710	B*3930					B*2701	B*3928			
B*1518	B*1537	B*2713	B*3932					B*2711	B*3933			
B*2702	B*1547	B*2715	B*4012					B*2714	B*3934			
B*2703	B*1549	B*2717	B*4802					B*2719	B*4440			
B*2704	B*1551	B*2725	B*4803					B*2720	B*4807			
B*2705	B*1552	B*2728	B*4804					B*2721	B*4808			
B*2706	B*1554	B*3805	B*4805					B*2724				
B*2707	B*1561	B*3809	B*4809					B*2727				
B*2709	B*1562	B*3810	B*4810					B*2730				
B*3801	B*1569	B*3811	B*4811					B*3526				
B*3901	B*1572	B*3904	B*4812					B*3903				
B*3902	B*1574	B*3907	B*4813					B*3905				
B*3909	B*1580	B*3914	B*9503					B*3906				
B*4801	B*1590	B*3918						B*3908				
B*7301	B*1591	B*3923						B*3911				
B44												
B*1801	B*1553	B*1820	B*4029	B*4056	B*4422	B*4435	B*5001	B*1546	B*4028	B*4048	B*4420	
B*3701	B*1803	B*3704	B*4035	B*4057	B*4424	B*4436	B*5002	B*1802	B*4030	B*4051	B*4425	
B*4001	B*1805	B*4005	B*4039	B*4102	B*4426	B*4437	B*5004	B*1814	B*4033	B*4052	B*4431	
B*4002	B*1806	B*4011	B*4040	B*4103	B*4427	B*4438		B*4003	B*4034	B*4058	B*4434	
B*4006	B*1810	B*4014	B*4049	B*4404	B*4428	B*4503		B*4004	B*4036	B*4059	B*4439	
B*4402	B*1811	B*4015	B*4050	B*4407	B*4429	B*4504		B*4009	B*4038	B*4101	B*4441	
B*4403	B*1813	B*4016	B*4053	B*4413	B*4430	B*4505		B*4010	B*4042	B*4104	B*4442	
B*4501	B*1815	B*4020	B*4054	B*4416	B*4432	B*4507		B*4018	B*4043	B*4106	B*4502	
	B*1819	B*4026	B*4055	B*4421	B*4433	B*4904		B*4019	B*4044	B*4107	B*4704	
								B*4023	B*4045	B*4405	B*4705	
								B*4024	B*4047	B*4414		
B58												
B*1516	B*1567	B*5804						B*5704				
B*1517	B*1595	B*5806						B*5705				
B*5701	B*5703	B*5807						B*5706				
B*5702	B*5707	B*5808						B*5805				
B*5801	B*5708	B*5809										
B*5802	B*5709	B*5811										
B62												
B*1501	B*1505	B*1530	B*1548	B*1578	B*1596	B*5202	B*9502	B*1504	B*1558			B*1309
B*1502	B*1514	B*1531	B*1550	B*1581	B*1597	B*5203	B*9504	B*1507	B*1573			B*1313
B*1512	B*1515	B*1533	B*1560	B*1582	B*3528	B*5204		B*1524	B*1586			
B*1513	B*1519	B*1534	B*1563	B*1583	B*4021	B*5205		B*1535	B*4408			
B*4601	B*1520	B*1538	B*1565	B*1585	B*4603	B*5207		B*1542	B*4602			
B*5201	B*1525	B*1539	B*1570	B*1588	B*4604	B*5208		B*1545				
	B*1528	B*1540	B*1575	B*1592	B*4605	B*7805		B*1555				
Unclassified												
B*0708	B*0806	B*1310	B*1543	B*1589	B*2723	B*3547	B*3709	B*4008	B*4410	B*4901	B*5402	B*8201
B*0710	B*0810	B*1311	B*1544	B*1804	B*2726	B*3548	B*3802	B*4013	B*4411	B*4902	B*5405	B*8202
B*0711	B*0814	B*1312	B*1556	B*1807	B*2729	B*3549	B*3803	B*4025	B*4412	B*4903	B*5512	B*8301
B*0713	B*0817	B*1404	B*1557	B*1808	B*3510	B*3550	B*3804	B*4027	B*4415	B*5107	B*5516	B*9501
B*0727	B*1301	B*1506	B*1564	B*1809	B*3513	B*3552	B*3808	B*4037	B*4417	B*5115	B*5606	
B*0728	B*1302	B*1511	B*1566	B*1812	B*3516	B*3556	B*3912	B*4046	B*4418	B*5122	B*5607	
B*0729	B*1303	B*1521	B*1571	B*1818	B*3519	B*3702	B*3919	B*4060	B*4506	B*5206	B*5608	
B*0732	B*1304	B*1527	B*1576	B*2708	B*3520	B*3705	B*3920	B*4061	B*4701	B*5303	B*5614	
B*0738	B*1306	B*1529	B*1577	B*2712	B*3523	B*3706	B*3922	B*4105	B*4702	B*5305	B*5901	
B*0804	B*1307	B*1532	B*1584	B*2716	B*3525	B*3707	B*3931	B*4202	B*4703	B*5307	B*6702	
B*0805	B*1308	B*1536	B*1587	B*2718	B*3527	B*3708	B*4007	B*4409	B*4806	B*5309	B*7803	

Figure 9: Supertype classification of HLA-A (top) and HLA-B (bottom) alleles. The alleles associated with each HLA-A supertype, multiple supertypes, or unclassified, are shown. Under each supertype, alleles are group by color on the basis of the stringency of selection: experimentally established motif (green), exact match/es in the B and F pockets (white), one exact and one key residue pocket match (yellow), key residue match/es at B and F pockets (grey). Alleles with no match at one or both pockets are listed with red font. Image from Sidney et al, BMC Immunology 2008 [86].

HIV infection induces a strong T cell mediated immune response, which can be readily detected activity *ex vivo* and quantified by several assays. Most commonly used assays include the gamma interferon (IFN-γ) enzyme-linked immunospot assay (ELISpot) [87], intracellular cytokine staining (ICS) [88] and suppression

capacity assays [89,90]. Together, these assays give an indication of the quantity and quality of the T cell responses. ELISpot assay is a sensitive and specific tool to determine T-cell frequency of antigen-specific T cells. This method is widely used in characterization of vaccine-induced responses since it has suitable characteristics for high-throughput screenings: is easy to perform, does not require expensive instrumentation and is highly sensitive [91]. ELISpot provides the magnitude of antigen-specific T cell responses, the breadth (total number responses to different HIV targets) and also the functional avidity of such responses, when determining the peptide concentration required to induce half-maximal responses. For functional information on cytokine production, definition of T cell phenotype, activation status and also proliferative capacity, the technique that is used is flow cytometry. In order to better describe the population and polyfunctionality of HIV-specific T cells, surface and intracellular staining of a variety of T cell markers is performed after incubation with antigen contact. A more recent *ex vivo* assay has been shown to predict clinical state of individuals and to distinguish HIV-controllers from progressors [90,92,93] and has been proposed to become a standard functional assay for immune readouts in clinical trials [94–96]. In summary, ELISpot is the most commonly used assay for quantification of CTL responses in clinical trials, being very sensitive and well established. However, other less standardized assays need to be taken in consideration when addressing more comprehensive questions regarding the functionality and type of response induced by a given intervention.

1.5.4 B cell response

After the initial $CD8^+$ T cell specific response, antibody activity agaisnst HIV-1 antigens can also be detected. A study of acute HIV-1 infection showed that early antibodies secreted were IgM, IgG and IgA subtypes and were specific for the Gp41 and Gp120 proteins [97]. However, none of the early antibody responses are able to effectively neutralize HIV-1 replication [98], due to the constant changes of the highly glycosylated viral envelope that HIV-1 presents [99]. Development of neutralizing antibodies able to neutralize multiple HIV-1 viral strains occurs months after infection, but only approximately 10-30% of chronically infected individuals develop broadly neutralizing antibodies (bNAbs) [100,101]. A number of clonal bNAbs were characterized as the first generation of antibodies including b12, 2F5, 4E10, 2G12 and Z13 [102]. The research for

new bNAbs increased after it was demonstrated that passive transfer of antibodies in rhesus macaques protected against SIV challenges [103,104]. The second generation of antibodies able to neutralize HIV-1 emerged soon, including 10-1074, VRC01, 3BNC117 or 10E8 [105] among an ever growing list. The isolation of such bNabs has been possible due to advances in improved methods for antibody isolation, single cell antibody cloning techniques [106] or structure-based design of engineered proteins [107]. The target regions of bNabs are mainly five (Figure 10): CD4 binding site, V1V2apex, glycan V3, fusion peptide (FP), Gp120-Gp41 interface and the membrane-proximal external region (MPER). Most of the bNAbs have been isolated from HIV-infected individuals, but until now, there is no vaccine immunogen design able to induce these broadly neutralizing antibodies *in vivo.*

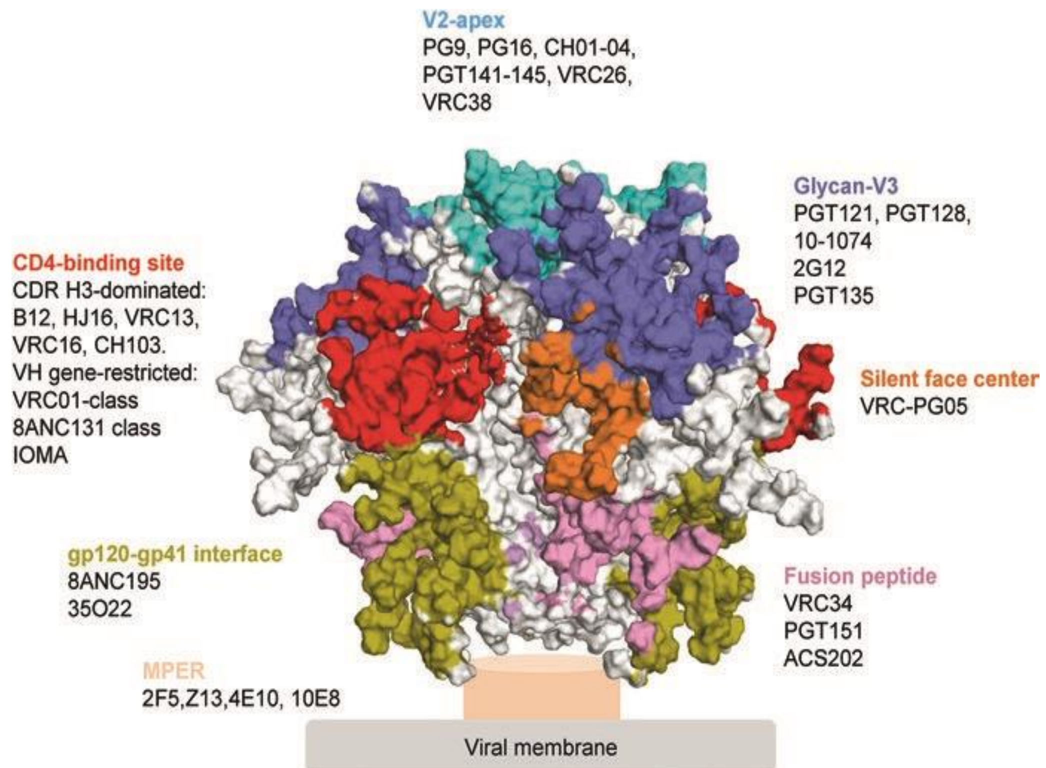

Figure 10: Main target regions of neutralizing antibodies. Major sites of vulnerability to neutralizing is shown, with a list of bNAbs targeting each one of the regions. Image from Tongqing Zhou and Kai Xu [108].

2. Natural control of HIV-1 infection

Few HIV-1 infected individuals are able to spontaneously control HIV-1 infection and do not develop HIV-1 disease, which represent between a 5-8% of the total HIV-1 infected population. They are referred to as long-term non progressors (LTNP) or, depending on the levels or viremia they present with, they are considered viremic controllers (VC), with detectable viremia up to 2,000 copies of HIV-1 RNA per milliliter of blood, or elite controllers, which represent less than 1% of all HIV-infected individuals (EC), with viral loads undetectable or up to 50 copies per milliliter of blood depending on the cut-off set by different viral load assays used. HIV-1 controllers are of great interest since understanding the basis of control in these individuals may help to define the key factors needed when designing a curative strategy. So far, several factors have been described to play a role in HIV-1 control, including a number of host genetic and viral factors [109,110]. Similarly, individuals that are multiply exposed to HIV-1 but remain uninfected (HESN), such as sex workers or serodiscordant sexual couples [111,112], can also help define correlates of protection to HIV-1 infection.

2.1 Host genetic markers

Over the last 30 years, large amount of host genetic markers have been described and associated with either rapid or slow progression of HIV-1 disease. However, the search for genetic markers involved in HIV-1 disease control needs to take into consideration the viral diversity in different host genetic settings, which depend on ethnicity or geographical diversity. In fact, it has been shown that different adaptation to the host genetics can lead to different alleles providing HIV control in geographically different sites [113]. Many host genetic polymorphisms associated with disease outcome are genes encoding for cell-surface proteins involved in innate or acquired immunity such as CCR5, human leukocyte antigen (HLA) or killer immunoglobulin-like receptors (KIR).

2.1.1 Association of HLA polymorphisms and disease progression

As mentioned, the high number of HLA class I alleles for the HLA-A, -B and -C loci results in T cell responses with many different epitope-specificities among individuals and leading to more or less efficient inhibition of HIV-1 replication [114,115]. To date, the genetic factors with the most profound impact on HIV

control are the HLA-B alleles. Particularly, alleles included in HLA-B7 (B*5101, B81), HLA-B27 (HLA-B27, B*1503) and HLA-B58 supertypes (HLA-B57, B*5801, B*1516, B*1517) have been associated with HIV-1 control and slow disease progression [116,117]. On the contrary, some other alleles, such as different HLA-B*35 alleles, have been associated with a faster disease progression [115,118]. According to peptide-binding specificity, HLA-B*35 subtypes are divided into two groups: HLA-B*35-Py group, which binds epitopes with proline in position 2 and tyrosine in position 9 and consists basically of HLA-B*3501; and the more broadly reactive HLA-B*35-Px group, which also binds epitopes with proline in position 2 but can bind several different amino acids (not including tyrosine) in position 9 [118]. Indeed, it has been suggested that B*35-Px allele (HLA-B*3502/3503/3504) but not B*35-Py alleles (B*3501) are associated with accelerated disease progression [118] because of a reduced capacity to present HIV-1 peptides. Less is known about the relationship between HLA class II polymorphisms and HIV-1 outcome, although recent studies have linked disease control to virus specific $CD4^+$ T cell responses and some specific alleles [119,120]. It has also been demonstrated that responses restricted by the same HLA can be associated with both reduced viral load and disease progression, which is the case of B*1503, associated with reduced viral loads in clade B infected individuals but not in clade C population [113]. Finally, in addition to certain HLA alleles being related with disease outcome, homozygous expression of HLA alleles has been linked to reduced viral control [121].

The protective effect of HLA-B57 and HLA-B27 has been associated with the presentation of multiple highly conserved and immunogenic epitopes, especially from Gag [116,122]. In fact, the broad epitope repertoire and wide cross-reactivity of HLA-B57 [123,124] suggest that the virus cannot escape efficiently from CTL HLA-B57 restricted responses. Viral escape occurs during acute infection following the apparition of CTL response, coincident with the viral load decline [125]. Complete escape from the first HIV-1 specific response can occur as early as 10 days after the first T cell response detection [126]. During the course of HIV-1 infection, the strong selection pressure on the virus continues, which can end up favoring mutations that can escape the CTL immune surveillance. However, the accumulation of such mutations has been shown to decrease the viral replication capacity of those escaped mutated viruses [119]. Hence, the global adaptation of HIV-1 to HLA class I polymorphisms and the presence of

mutated impaired viruses in plasmas of HIV controllers [127] supports the key role of HIV-specific CTLs in HIV-1 infection.

Subsequent studies on the adaptation of HIV-1 to human leukocyte antigen class I have been performed in order to define which mutations occur upon certain HLA-restricted CTL pressure [114,128–130]. HLA-associated polymorphisms identified by statistical associations are commonly displayed in immune-escape maps as shown in Figure 11 [130]. On the one hand, non-adapted associations are HIV-1 amino acids that are under-represented among persons expressing the HLA allele in question, thus representing the inferred immunologically susceptible forms of the epitope. On the other hand, adapted associations are amino acids residues enriched among persons expressing the specific HLA allele, representing the inferred escape forms particular to that epitope/HLA complex. Such escape mutations, predicted by HLA restriction of the specific epitope, are also termed HLA "footprints".

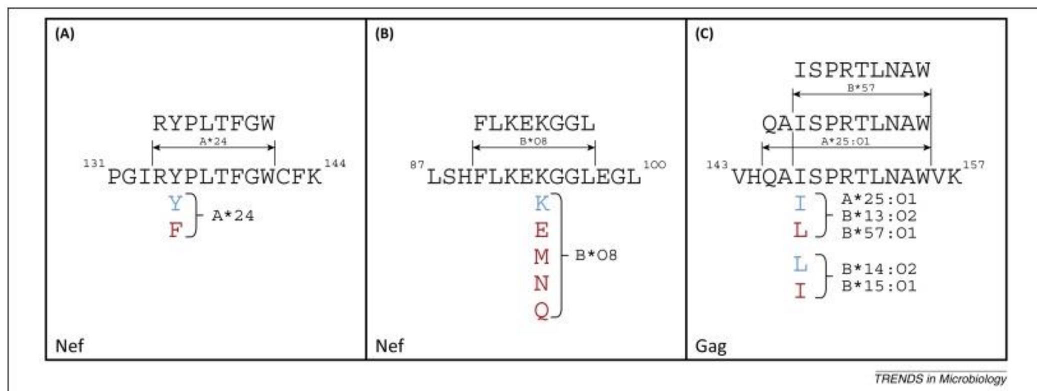

Figure 11: Examples of HLA-associated polymorphisms identified in HIV-1. The HIV-1 subtype B consensus sequence is used as a reference, known HLA-restricted CTL epitopes are marked above and HLA-associated polymorphisms below the epitope sequence. Non-adapted associations are shown in blue and adapted associations are shown in red. Figure A shows the case of the HIV-1 Nef codon 135, located at position 2 of the HLA-A*24-restricted RW8 epitope. Residues Y and F represent the non-adapted (susceptible) and adapted (escaped) forms associated with HLA-A*24, respectively. In B, a single HLA allele driving multiple possible escape pathways at a single site is shown. At Nef codon 94, position 5 of the B*08-restricted FL8 epitope, the B*08-associated non-adapted form is the subtype B consensus K, but there are four possible adapted forms: E, M, N, and Q. In Figure C, codons under pressure by multiple HLA alleles are shown. At Gag codon 147, the adapted form associated with HLA-A*25:01, B*13:02, and B*57:01 is L, whereas the adapted form selected by HLA-B*14:02 and B*15:01 is the subtype B consensus I. Figure from Carlson et al, Trends in Microbiology 2014 [130].

Aside from polymorphisms in the MHC region, KIR receptors have also been associated with increased or decreased severity of HIV-1. Since functional interaction can only occur when both ligands and receptors are co-expressed in the same individual, responses due to this interaction widely vary depending on

the individuals' genetics. Distinct allele combinations of those genes seem to be more or less favorable than others in different specific diseases, including HIV-1 replication *in vivo*. For instance, co-expression of KIR3DS1 and HLA-Bw4 has been associated with slow progression and low viral load in untreated chronic HIV-1 infection [131,132].

2.1.2 HLA independent genetic markers of HIV control

Another key molecular family associated with HIV-1 control is the superfamily of 7-transmenmbrane G-protein-couple chemokine receptors [133]. Two coreceptors of this family, CCR5 and CXCR4, are the main receptors that HIV-1 uses to enter target cells [134], although less frequent coreceptors can also be used by particular viruses. The distinction of viral tropism relies on the chemokine receptor that viruses use to enter the target cell, being R5-tropic virus the ones that use CCR5, X4-tropic viruses the ones that use CXCR4, and R5/X4 virus the ones that can use both of these receptors, expressed by T cells and macrophages [135]. One genetic polymorphism in CCR5, a 32-base-pair deletion (CCR5-Δ32) was reported to generate a non-functional protein. As CCR5-Δ32 cannot be expressed on the surface of the cell, HIV-1 cannot enter the target cell. Therefore, homozygous expression of CCR5-Δ32 is related to resistance to infection with R5-tropic HIV-1 [136]. Heterozygous expression of wildtype CCR5/CCR5-Δ32 does not provide protection but results in lower levels of CCR5 expressed in the membrane, associated with reduced viral load and delayed disease progression [136–139]. Nevertheless, this is not a common scenario given that the frequency of homozygosity for this deletion is predominantly found in Northern European populations with a median frequency of CCR5-Δ32 allele distribution of 10% and less than 1% of homozygosis for CCR5-Δ32 genotype frequencies [140]. For this reason, it is of great interest to compile information on CCR5Δ32 genotypes from donors in biobank systems, to which can be critical for those particular HIV infected individuals that eventually require a bone marrow transplant due to an hematological disease. In addition to CCR5-Δ32 mutation, other mutations have been related with reduced R5-tropic HIV-1 infection, such as mutations in the coreceptor CCR2, the 3' untranslated region of the SDF-1 chemokine gene and other promoter polymorphisms responsible for higher expression of RANTES/CCL5, MIP-1α/CCL3L1, MIP-1β, MIP1αP and DC-SIGN [141].

2.2 Viral determinants

While immune and host genetic parameters associated with viral control in infected individuals have been widely studied, less is known about viral determinants that could be driving control of HIV infection. Viral replication capacity and cell tropism have been linked to HIV-1 protection from disease progression, although the latter has not been consistently documented [142,143]. Some existing viral isolates have been shown to be defective, being less infective and therefore, are considered to contribute to a controller phenotype [144,145]. Actually, some studies identified individuals or small cohorts such as the Sydney Blood Bank Cohort (SBBC), that harbor this type of defective virus with lower replication capacity [146]. In this particular cohort, 8 individuals were infected from a single donor, with an attenuated, nef/LTR-deleted strain, which resulted in either low or absent *in vivo* viral replication for up to 29 years [147]. In fact, more cases of mutations in Nef have been described in different clinical studies, and have been associated with maintained viral suppression for long periods of time in the recipients carrying the mutated virus [148–151]. The suggested mechanisms in those cases is the inability of the mutated Nef protein to down-regulate MHC class I molecules, rendering infected cells more susceptible for HIV-specific CTL [152]. However, longitudinal analysis on SSBC individuals showed an evolution of the nef/LTR sequences towards a stable protein, suggesting that the deletions of nef sequence was not fundamentally mediating the longitudinal protection presented by this cohort [153]. Other mutations in proviral structural and accessory proteins have also been associated with slower disease progression, including mutations, deletions and/or insertions in Gp41, Gag or Vpu [154–156]. Nevertheless, despite these reported cases of reduced replicative viruses, it is still unclear to what degree the viral diversity contributes to a controller phenotype and what the most critical sites in the virus are that define viral fitness [157].

3. Strategies to find and HIV cure

The success of antiretroviral therapy has completely changed the course of the AIDS pandemics, transforming HIV to a chronic manageable disease in those places where ART is avaliable. Since 1986, when the first antiretroviral drug (azidothymidine, AZT) was described [158], several drugs targeting different HIV cell cycle steps have been developed. The introduction of antiretrovirals in 1996, specially highly active antiretroviral treatment (HAART), reduced mortality rates by 80-90% over the past decades. To date, more than 30 drugs have been approved, classified based on their molecular mechanism into: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors (InIs), fusion inhibitors and co-receptors antagonist. Combined antiretroviral therapy is able to efficiently suppress viral replication and reduce the plasma HIV-1 viral load below limits of detection, reconstituting the immune system [159–161] and avoiding progression to AIDS. Currently, 21 millions of people living with HIV are actively treated and receive therapies consisting of combinations of 2 or 3 drugs directed towards at least two distinct molecular targets in order to avoid the appearance of drug resistances and clinical drug failure [162–164]. Moreover, in recent studies of thousands of serodiscordants couples, there was no sexual transmission of HIV from a virally suppressed person to their seronegative partners, pointing out the role of cART in preventing transmission to others [165,166]. Guidelines for the use of antiretroviral agents are available and updated every year, covering information about new drugs under development, new recommendations of drug combinations and regimen simplifications [167,168]. Figure 12 shows all the drugs available and approved for its clinical use, categorized by drug class, as well as pills containing combination of 2 or more drugs. Finally, antiretroviral drugs are also successfully used for post-exposure prophylaxis and more recently, as pre-exposure prophylaxis (PrEP) in HIV-negative people at high risk of HIV acquisition [169].

Nucleoside-analog reverse transcriptase inhibitors (NRTIs)

▼**DESCOVY®**
(emtricitabina 200 mg/ tenofovir alafenamida 10 o 25 mg, DVY)
Gilead Sciences

TRUVADA®
(emtricitabina 200 mg / tenofovir disoproxilo 245 mg, TVD)*
Gilead Sciences

VIREAD®
(tenofovir disoproxilo 245 mg, TDF)*
Gilead Sciences

EMTRIVA®
(emtricitabina 200 mg, FTC)
Gilead Sciences

Epivir®
(lamivudina 300 mg, 3TC)
ViiV Healthcare

Kivexa®
(abacavir [como sulfato] 600 mg / lamivudina 300 mg, KVX)
ViiV Healthcare

Videx®
(didanosina 2 g polvo para solución oral, DDL)
Bristol-Myers Squibb

Ziagen®
(abacavir [como sulfato] 300 mg, ABC)
ViiV Healthcare

Combivir®
(zidovudina 300 mg / lamivudina 150 mg, CBV)
ViiV Healthcare

Retrovir®
(zidovudina 250 mg, AZT)
ViiV Healthcare

Trizivir®
(zidovudina 300 mg / lamivudina 150 mg / abacavir [como sulfato] 300 mg)
ViiV Healthcare

Fusion inhibitors

Fuzeon®
(enfuvirtida 90 mg/ml polvo y disolvente para solución inyectable, ENF, T-20)
Roche

Co-receptors antagonists

Celsentri®
(maraviroc 150 mg o 300 mg, MVR)
ViiV Healthcare

Integrase inhibitors

▼**BIKTARVY®**
(bictegravir 50 mg / emtricitabina 200 mg / tenofovir alafenamida 25 mg, BVY)
Gilead Sciences

▼**GENVOYA®**
(elvitegravir 150 mg / cobicistat 150 mg / emtricitabina 200 mg / tenofovir alafenamida 10 mg, GNV)
Gilead Sciences

STRIBILD®
(elvitegravir 150 mg / cobicistat 150 mg / emtricitabina 200 mg / tenofovir disoproxilo 245 mg, STB)*
Gilead Sciences

Triumeq®
(dolutegravir 50 mg / abacavir 600 mg / lamivudina 300 mg, TMQ)
ViiV Healthcare

▼**Juluca®**
(dolutegravir 50 mg / rilpivirina [como hidrocloruro] 25 mg, JLC)
ViiV Healthcare

Tivicay®
(dolutegravir 50 mg, DTG)
ViiV Healthcare

Isentress®
(raltegravir [como sal potásica] 600 mg o 400 mg, RAL)
MSD

Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

▼**ODEFSEY®**
(tenofovir alafenamida 25 mg/ emtricitabina 200 mg / rilpivirina [como hidrocloruro] 25 mg, ODE)
Gilead Sciences

EVIPLERA®
(rilpivirina [como hidrocloruro] 25 mg / emtricitabina 200 mg / tenofovir disoproxilo 245 mg, EPA)*
Gilead Sciences

ATRIPLA®
(efavirenz 600 mg / emtricitabina 200 mg / tenofovir disoproxilo 245 mg, ATP)*
Gilead Sciences y Bristol-Myers Squibb

Sustiva®
(efavirenz 600 mg, EFV)
Bristol-Myers Squibb

Edurant®
(rilpivirina 25 mg, RPV)
Janssen-Cilag

Viramune®
(nevirapina 200 mg o 400 mg, NVP)
Boehringer Ingelheim

Intelence®
(etravirina 200 mg, ETR)
Janssen-Cilag

Protease inhibitors

▼**Symtuza®**
(darunavir [como etanolato] 800 mg / cobicistat 150 mg / emtricitabina 200 mg / tenofovir alafenamida [como fumarato] 10 mg, STZ)
Janssen-Cilag

Reyataz® + Norvir®
(atazanavir 300 mg + ritonavir 100 mg, ATV + RTV)
Bristol-Myers Squibb / AbbVie

▼**Evotaz®**
(atazanavir 300 mg / cobicistat 150 mg)
Bristol-Myers Squibb

Prezista® + Norvir®/Tybost®
(darunavir 400 mg o 800 mg + ritonavir 100 mg o cobicistat 150 mg, DRV + RTV o C)
Janssen-Cilag / AbbVie / Gilead Sciences

▼**Rezolsta®**
(darunavir [como etanolato] 800 mg / cobicistat 150 mg)
Janssen-Cilag

Prezista® + Norvir®
(darunavir 600 mg + ritonavir 100 mg, DRV + RTV)
Janssen-Cilag / AbbVie

Aptivus® + Norvir®
(tipranavir 250 mg + ritonavir 100 mg, TPV + RTV)
Boehringer Ingelheim / AbbVie

Invirase® + Norvir®
(saquinavir 500 mg + ritonavir 100 mg, SQV + RTV)
Roche / AbbVie

Kaletra®
(lopinavir 200 mg / ritonavir 50 mg, LPV / RTV)
AbbVie

Telzir® + Norvir®
(fosamprenavir cálcico 700 mg + ritonavir 100 mg, FPV + RTV)
ViiV Healthcare / AbbVie

Crixivan®
(indinavir 400 mg, IDV)
MSD

Figure 12: Guide of the approved drugs for the treatment of HIV in Spain, adapted from GILEAD 201

As mentioned, current antiretroviral treatment regimens are effectively able to suppress virus replication to undetectable levels but are not able to completely eliminate the viral reservoir and the HIV-1 proviral DNA remains integrated in the host genome. Therefore, interruption of treatment in individuals under cART leads generally to rapid viral rebound of plasma viremia within few weeks. Due to such limitation of cART, the search for alternative strategies for an HIV cure has gained strong interest in the HIV field over the past decade. Many strategies are being investigated in order to achieve either a functional cure of HIV-1 infected individuals (similar to EC phenotype) or a sterilizing cure (complete elimination of the virus). Several lines of research are still at preclinical level, but some strategies have been already tested at a clinical stage, including early treatment initiation and cART intensification, passive transfer of antibodies, therapeutic vaccination, kick and kill strategies, stem cell transplantation and gene therapy, among others.

3.1 cART intensification and early treatment initiation

Several clinical trials have tested whether the intensification of cART using additional antiviral drugs on top of standard regimens would be able to reduce the frequency of latently infected cells. However, none of them have shown any further suppression of low-level viremia nor reduction in the number of latently infected cells [170–174]. Of note, studies using the integrase inhibitor-based raltegravir detected the presence of residual low-level viral replication. Two randomized clinical trials showed a transient but significant increase in two-long terminal repeat (2-LTR) circles upon ART intensification, compared to placebo [170,171,175]. Using integrase inhibitor-based therapies such as raltegravir, HIV-1 DNA is not able to integrate into the host DNA and it circularizes forming 2-LTR circles. For this reason, detection of 2-LTR circles is now being considered a measure of residual ongoing replication. It is still debated though from which type of cells or tissues residual replication stems and whether it could be associated with the insufficient drug concentration in lymphoid tissues compared to peripheral blood mononuclear cells (PBMCs) [176]. Understanding the contribution of such ongoing residual replication during ART is of great interest for HIV cure strategies that aim to completely eliminate HIV latency.

In addition to cART intensification, early treatment initiation has been shown to limit seeding of the viral reservoir, resulting in lower levels of latently infected cells and, moreover, limiting the diversity of the viral reservoir population [177]. There is robust

evidence that the latent HIV reservoir is established very early after infection, within days after transmission [178,179]. In fact, "post-treatment controllers" (PTC) were described as individuals who received very early cART and showed immunovirological control after treatment cessation [180–183]. The first case of PTC was described in 2011 by Salgado et al, who reported a case of prolonged control of replication-competent HIV-1 for nine years after treatment cessation. This report suggested that some individuals were capable of controlling HIV-1 for extended periods of time through distinc mechanism from the ones reported in EC [184]. Later on, Sáez-Cirión et al presented the ARNS VISCONTI (from Virological and Immunological Studies in CONtrollers after Treatment Interruption) cohort in 2013 [181], consisting of a group of 14 early-treated HIV-infected individuals (now 23 individuals included) that received cART at latest during Fiebig V, for a median of time of 9 years and that maintained long-term viral control (average of 7 years) after treatment cessation. The same group reported a case of a perinatally infected child treated from birth for at least 5 years and that had a similar outcome when examined again at 18 years of age [185]. One year later, the "Mississippi baby" case was reported [186], where a baby was diagnosed as HIV positive at birth and treated with intense ART within 30 hours of age. After few months of cART cessation, at 18 months of age the baby had no detectable viremia. However, virus rebounded after 27 months off treatment. A recent metanalysis including over 700 individuals from 14 different interruption trials, has characterized the viral dynamics of 67 PTC. In this study, PTC were defined as individuals able to maintain plasma viremia <400 cop/ml for at least 24 weeks after cART was interrupted in >2/3 of determinations. With this definition, a 13% rate of PTC was estimated in early-treated individuals vs a 4% in individuals who started cART in chronic stage of HIV infection [183].

Of critical importance for cure strategies that aim to boost individuals' immune system by therapeutic vaccination, most PTC do not seem to have the same mechanism of control as "elite controllers" since they have poorer HIV-specific $CD8^+$ T cell responses and lower levels of $CD8^+$ T cells expressing $CD38^+HLA\text{-}DR^+$ [181]. However, PTC do have a very low reservoir after treatment, and a robust HIV-specific $CD4^+$ T cell responses [187]. To date, there is still little knowledge on the mechanism behind the post-treatment control and the role that previous ART plays in those cases. Actually, since the description of post-treatment phenotype, there is still the debate whether PTC are EC that could have controlled the virus even without a period of ART, or whether they are two groups with two different phenotypes [188].

It is however well established that early cART is associated with lower cells carrying replication-competent virus and HIV DNA levels [189–191]. Indeed, results from RV254/SEARCH010 studies, one of the best characterized and earliest cohorts in terms of acute HIV infection, demonstrated that there is progressive increase of the viral reservoir during the first few weeks of infection [192] and that early cART does limit the reservoir size compared to chronic treatment [179]. Finally, as early treated individuals show less exhaustion of their HIV specific T cell populations, fewer escaped virus variants and more effective T cell responses in addition to a lower reservoir [96,177,193,194], they are a population of great value when testing new HIV cure strategies.

3.2 Stem cell transplants and gene therapy

The first person who has been cured of HIV infection without any signs of virus presence has been described now for more than 10 years. This individual known as "the Berlin patient", received a bone narrow transplant containing stem cells from a matched donor homozygous for CCR5-Δ32 as a treatment for leukemia. ART discontinuation after stem cell transplant (SCT), resulted in the complete elimination of the virus [195]. A second case of CCR5-Δ32 donor stem cell transplant was reported recently, named "the London patient" [196]. In this case, the regimen before transplant was less aggressive than in the Berlin patient, without any irradiation and using a reduced intensity conditioning and T-cell depletion with αCD52 instead of ATG. Both patients presented a mild graft versus host disease and achieved 100% of T-cell donor chimerism. For this reason, stem cell transplantation from CCR5-Δ32 and CCR5 gene editing are one of the mechanisms being studied and tested as a cure strategy in a growing number of patients that need to undergo stem cell transplant as treatment for underlying cancer [197–200].

SCT has become a standard procedure to treat high-risk leukemia and lymphomas. This procedure results in a complete change of the circulating T cells, a unique opportunity to clear infected circulating as well as tissue T cells. Although SCT has achieved a replacement of circulating T lymphocytes in HIV^+ individuals, HIV can still persist unless stem cells are from a donor with CCR5 delta 32 mutation. This is highlighted by two cases of HIV remission (referred to as the Boston patients), who received SCT but without CCR5-Δ32 mutation. Although they showed an elimination of detectable latently infected cells, there was a viral rebound 12 and 32 weeks after treatment interruption, respectively [201]. Another case of allogenic stem cell

transplant was reported few years later, know as the Mayo clinic patient, with viral rebound after 10 months into treatment interruption [202]. Despite the fact that SCT in HIV-infected individuals represent the first cases of HIV cure, there is still a lot to be understood on these procedures. In particular, it is not known whether the stem cell procedure by itself, the CCR5 delta 32 mutation on the donor cells, the ablative procedure, the graft-versus-host reaction or the combination of those factors are responsible for driving HIV clearance. As SCT is only used to treat high-risk leukemia, these unique cases of HIV-infected patients undergoing SCT are being identify and follow-up to try to find the answer behind the mechanism of HIV cure in this setting. Actually, the observational project ICISTEM who reported the London patient, is investigating more cases of allogeneic stem cell transplant in HIV-1-infected individuals in Europe.

The discovery of the resistant phenotype of CCR5-Δ32 homozygous mutation and the "Berlin patient" case raised strong interest towards the use of genetic engineering to silence or disrupt the CCR5 gene. Research on gene therapy, using techniques like zinc fingers nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and most recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system [203] has progressed as well. However, the field is still in its early stages and the only system that has progressed to clinical trial is ZFN. The clinical trials performed (NCT00842634, NCT01044654), consisted on harvesting autologous cells from HIV-infected individuals, modifying them *ex vivo* with ZFN method, and then reintroduce CCR5-depleted ZFN-modified cells into the individual [197,204]. Phase I studies showed that the administration of *ex vivo* genetically modified autologous $CD4^+$ T cells was safe and that cells were detectable even after 42 months after reinfusion, although at low frequencies [197]. In addition, and despite the fact that modified $CD4^+$ T cells were present in the blood, the intervention was not capable to delay viral rebound when cART was interrupted. This outcome may be explained by the low percentages of modified cells (less than 10%) compared to non-modified cells, still leaving ample target cells with intact CCR5 receptors. A recent study using CRSIPR-based genome editing showed similar results with only 5% of disrupted CCR5 lymphocytes after transplantation of CRISPR-*ex vivo* edited CCR5-ablated hematopoietic stem and progenitor cells (HSPCs) (NCT03164135) [205]. A phase II clinical trial is now on going (NCT02500849) where the same ZFN mechanism is used to modify hematopoietic stem cells, instead of T cells. Results from this clinical trial, and other gene therapies that might be tested,

will give further insights on the safety, cost and the hurdles that such approaches may face if they should be implemented as a common cure strategy.

3.3 Antibodies

Broadly neutralizing antibodies have been shown to accelerate clearance of cell-free virus [197], induce ADCC activity [206] and activate macrophages and NK cells *in vivo* [207]. Moreover, bnAbs induce the formation of immune complexes with viral particles, improving antigen presentation to T cells, and therefore increasing T cell activity [207]. Therefore, it is believed that an effective vaccine against HIV-1 would require elicitation of bnAbs. Nevertheless, bNAbs described so far present such high level of somatic mutations [208], that generating a vaccine able to induce them is extremely difficult and would require a sequential immunization to direct the immune system towards those changes [209]. While the knowledge of these strategies on active immunization are still growing, passive immunization by transfer of antibodies has been proposed as an HIV cure strategy. Administration of bNAbs can be used for passive protection, post-exposure prophylaxis, chronic therapy and eradication [210]. Completed clinical studies of passive administration of antibodies performed so far are listed in table below (Table 3). In fact, some of the studies have shown suppressed viremia during the antibodies infusion [211–213] and delayed viral rebound [214]. However, one of the major limitations of bNAbs is that when given in monotherapy, viral variants resistant to the antibody appear readily [212]. As a consequence, new ongoing phase I and phase II clinical trials include combinations of two bNAbs. The only completed clinical trial using two broadly neutralizing antibodies (NTC02825797) showed a delayed viral rebound to a median of 21 weeks and showed no signs of virus resistance to eitherantibody 3BNC117 and 10-1074 [215]. Ongoing trials include new regimens and new antibodies, such as 10-1074-LS an 3BNC117-LS (NCT03554408) or 3BNC117 and 10-1074 (NCT03875209, NTC03526848), or combination of bNAbs with latency-reversing agents such as 3BNC117 and romidepsin (NCT02850016) or lefitolimod (TLR9 agonist) and 3BNC117/10-1074 (NCT03837756), among others. These new trials can be expected to clarify whether bNAbs (alone or in combination with other therapies) are able to reduce or completely eliminate the latent reservoir and control HIV in absence of cART treatment.

Antibody	Trial design and size	Effect	References
CHERUB 001 Intravenous immunoglobulin (IVIG)	Controlled trial in acute infection, 5 days 30g IVIG, n=10. No ATI	No changes in proviral HIV-1 DNA	Tiraboschi et al 2017 [216]
3BNC117 bnAb - (NTC02018510)	Single infusion 30mg/kg in HIV-1 infected individuals	Reduced viral load for 28 days after infusion	Caskey et al 2015 [213]
- (NCT02446847) (targets the HIV CD4-binding site)	2 infusions (30 mg/kg) separated by 3 weeks or 4 infusions separated by 2 weeks n=13, ATI	Significant delayed rebound during ATI in both regimens	Scheid et al 2016 [214]
10-1074 bnAb (targets the V3 glycan supersite on Env) (NTC02511990)	Single infusion 30 mg/kg (n=13)10 mg/kg, (n=3) and 3mg/kg (n=3) in mostly off-ART individuals	Decreased viremia in individuals off-cART after receiving 30mg/kg dose Viral rebound after 3-4 weeks after infusion Emergence of multiple resistant viruses	Caskey et al 2017 [212]
3BNC117 + **10-1074** bNAbs (NTC02825797)	3 infusions every 3 weeks of 30mg/kg per antibody, n=9. ATI after first infusion	Delayed viral rebound to a median of 21 weeks No signs of viruses resistant to 3BNC117 and 10-1074	Mendoza et al 2018 [215]
VRC01 (targets the HIV CD4-binding site) - (NCT01950325)	Two infusion doses 1mg/kg (n=3), 5mg/kg (n=7), 20mg/kg (n=5) and 40mg/kg (n=12) including treated and untreated HIV-1 individuals	Plasma viremia was decreased in untreated individuals after infusion. No changes in HIV-1 DNA proviral Emergence of virus non sensitive to antibody neutralization	Lynch et al 2015 [217]
- (NCT02463227)-(ACTG) A5340	3 doses 40mg/kg every 3 weeks, starting 8-week ATI after 1 week of the 1st dose, n=14	No delay on viral rebound Emerging antibody neutralization–resistant virus	Katharine et al 2016 [218]
- (NCT02471326) - NIH 15-I-0140	8 doses of 40mg/kg , 1st before starting 6-month ATI; 2,4 and 8 weeks into ATI and 4 monthly doses after cART resumption, n=10	Modest delay on viral rebound compared to historical controls Sensitivity diminished significantly after multiple infusions	Katharine et al 2016 [218]
- NCT02664415/ NCT03036709	placebo-controlled (3:1) trial in acute infection, receiving during ATI 8 doses of 40mg/kg every 3 weeks, n=18	Minimal delayed viral rebound	Crowell et al 2019 [219]
UB-421 (inhibitor of HIV binding to CD4 receptors) (NCT02369146)	During ATI, 8 infusions of 10mg/kg every week n=14, or 8 infusions 25mg/kg every 2 weeks, n=15	Virology suppression was maintained (<20cp/mL) during the antibody administration	Wang et al 2019 [211]

Table 3: Summary of clinical trials using antibodies.

3.4 Therapeutic vaccination

Therapeutic vaccination aims to improve the HIV-specific immune responses in HIV-infected individuals, in order to achieve long term remission or even clearance of all infected cells. To generate such HIV specific immune enhancement, multiple strategies are being studied, in particular T cell-based vaccines. As mentioned before, CTL play a crucial role in the initial decay of viremia after reaching the acute peak of VL, and parameters related to antiviral T cell responses have been strongly correlated with controlled HIV infection. These observations have led to the development of a wide range of T-cell vaccines, some of them already tested in clinical trials either with HIV uninfected or HIV infected individuals. Although some of these studies have shown an increase in the number and function of HIV-specific cells post vaccination [96,212,214,220–225], none has proven to mediate efficient long-term *in vivo* control of viral replication in the absence of antiretroviral therapy. The lack of effect is probably due to a number of factors, including the failure of current vaccines and vaccination strategies to elicit broad, potent and polyfunctional T cell responses able to prevent viral escape during viral rebound. Importantly, vaccine-induced responses, even of the right functional phenotype, may also be ineffective due to their inability to recognize of recognizing latently infected cells, highlighting the potential need for combination approaches that combine vaccines with strategies to reactivate the latent viral reservoir (see below).

Vaccine immunogens based on consensus HIV sequences and directed towards one sole protein such as Gag, ended up eliciting narrow and few T cell responses that the virus is able to escape by mutation of single or few amino acid residues [224–226]. Therefore, inducing superior cellular immunity against HIV-1 may require the generation of either a broad response, or a response directed towards more susceptible viral targets. To date, the clinical vaccine trial that has demonstrated a partial efficacy in virus control post cART interruption was based on using antigen-pulsed autologous dendritic cells (DC-HIV) that elicited broad T-cell responses against Gag, Nef and Env and which resulted in a 10-fold transient reduction of the viral set-point after treatment discontinuation [227].

One strategy used to overcome limitations in immunogen design is by basing the design of the vaccine immunogen on the most conserved regions of the HIV genome, in which mutational escape might be more challenging. One of this design, the p24CE immunogen (NCT03181789) express 7 conserved elements of the Gag p24 protein, which contains a high number of HLA class I and class II-restricted epitopes [228].

Another example of immunogen design based on conserved HIV sequences is the sequence referred to as HIVconsv [229,230].This immunogen has been developed at the University of Oxford and includes 14 conserved regions from the HIV Gag, Pol, Env and Vif protein sequences across all M group viruses (clades A, B, C and D) [229]. The HIVconsv has been already tested in several clinical trials in HIV-uninfected and HIV-infected individuals and using different vector platforms as delivery vectors, such as plasmid DNA and viral vectors including a replication defective simian adenovirus (ChAd) and a recombinant poxvirus (modified vaccinia Ankara, MVA) [96,231,232]. A newer version of this immunogen has been developed, the tHIVconsvX, which incorporates more functional immune data in its design and includes bivalent complementary mosaic immunogens to maximize global epitope coverage and which is hypothesized to block common escape pathways [230]. However, and despite capacity of induction of T cells with high inhibitory capacity [233] it has not yet been proven that vaccine-induced responses towards all these regions are of enough functionality to contain viral replication upon treatment interruption or have an impact on the viral reservoir. Another approach to design a T-cell vaccine immunogen in which our group has been focused, is by using functional human immune data rather than sequence alignments to inform immunogen design. As a consequence, this HIVACAT-T-cell immunogen (HTI) sequence only comprises beneficial regions identified by large-scale human immune screenings that allowed to classify HIV-1 viral targets able to induce responses into beneficial, neutral or non-beneficial in terms of associations with viral control [234]. Several clinical trials including these vaccine immunogens are currently ongoing and will help to clarify the importance of T cell specificity in immune-driven virus control.

Aside from the complexity of generating an immunogen design able to elicit broad and functional responses, vaccine delivery methods are another important consideration. HIV vaccines have been tested using various delivery methods such as replication-competent and replication-defective viral vectors, nucleic acids, proteins, and various adjuvants [235]. In fact, combinations with new adjuvants such as TLR agonist [236] and new vectors such as RNA [237] or cytomegalovirus (CMV) [238] are being tested. It has been described recently that vaccines using CMV-vectored SIV vaccines lead to SIV control and clearance after SIV challenges in half of the rhesus macaques that received the vaccine[239]. Although the mechanism of clearance has not been well defined, the hypothesis is that class II restricted $CD8^+$ vaccine-induced SIV-specific responses may mediate protection [240]. However,

controller and non-controller animals did not differ significantly in the number of such MHC class II restricted CD8+ T cell responses and the search for functional immune correlates of protection in this setting is ongoing, including an intensive analysis of T cell responses restricted by Mamu-E, the HLA-E homolog in humans. Results from ongoing and future studies are urgently needed to keep improving of T-cell immunogen design, vector choice, and vaccination regimen before attaining a successful therapeutic T cell vaccine strategy. Furthermore, it is likely that T cell vaccine would be used in combination of other strategies such as kick&kill strategies, presented in the following section.

3.5 Kick&kill strategies

The major obstacle for an HIV cure is the HIV reservoir, defined as the infected cell population that allows for life-long persistence of replication-competent HIV-1 in patients on optimal cART regimens [176]. Although cART is able to inhibit viral replication, is not able to purge the latent reservoir, and therefore latently infected cells retain the capacity to produce infectious virus when treatment is interrupted. These latently infected cells represent the major barrier to completely eradicate HIV-1. One therapeutic approach that targets HIV reservoir is the “kick and kill” strategy [241], which propose to use a latency reversing agent (LRA) to induce the transcription of viral genes from latently infected cells and render them susceptible to immune mediated-killing by cytolytic T lymphocytes (CTL) (Figure 13).

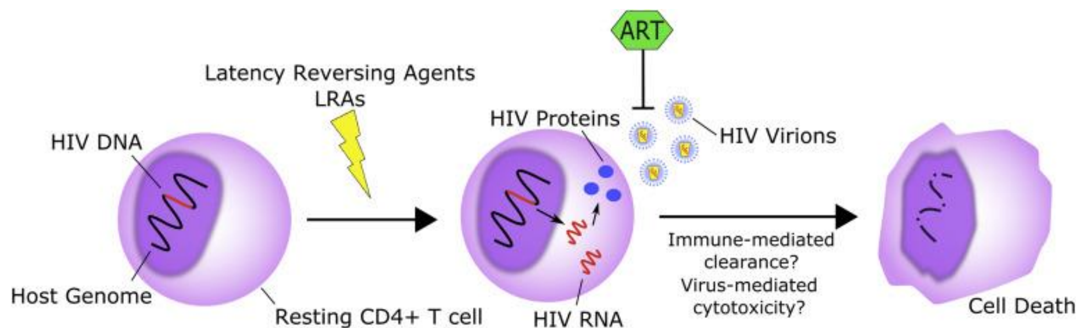

Figure 13: Kick and kill strategy to eliminate HIV latently infected cell. Image from Kim et al, Cell Host & Microbe 2018 [242].

Over the past decade, various LRAs have been found to induce viral gene expression from latency *in vitro*, including histone deacetylase inhibitors (HDACis), histone methyltransferase (HMT) inhibitors, DNA methyltransferase inhibitors, bromodomain inhibitors, protein kinase C (PKC) agonists, phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (Akt) pathway inhibitors and agonists of the innate

immune receptors TLR7 or TLR9 [243–245] as presented in Figure 14. Some of the most potent LRA have been tested in preclinical and in small-scale clinical trials. A summary of the clinical trials conducted so far with LRAs is shown in Table 3.

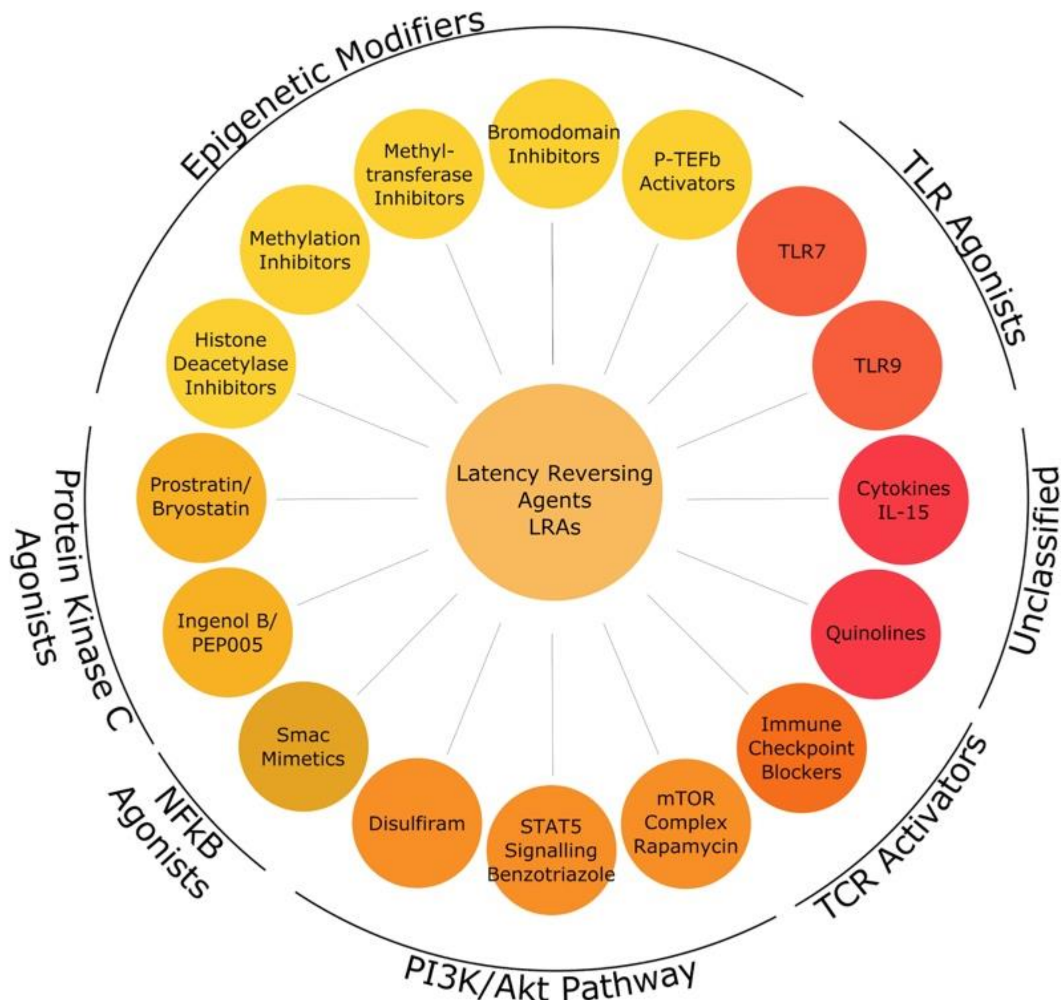

Figure 14: Classes of LRAs. LRAs can act on different pathways, resulting in an increase in HIV transcription and/or virion production. Image from Kim et al, Cell and Host Microbe 2018 [242].

One of the best studied family of LRA are histone deacetylation inhibitors (HDACi). As shown in Figure 15, inhibition of histone deacetylases (HDACs) by HDACi promotes histone acetylation by histone acetyl transferases (HATs) leading to relaxation of the chromatin and initiation of a generalized transcription, including HIV genes. It has been shown that in patients with HIV infection, the use of HDACi results in reactivation of the integrated HIV genes and viral protein expression by latently infected cells [153–155]. Some of the HDACi tested as LRA candidates such as vorinostat, panobinostat or romidepsin are approved by the Food and Drug Administration (FDA) for use in different types of cancer.

LRA	Trial design and size	Effect	References
Vorinostat (400mg)	Single-arm, single dose, n=8 Chronically ART-supressed	4.8_{fold} increased transcription, no change in proviral HIV-1 DNA	Archin et al 2012 [246]
	Single-arm, 14 daily infusion, n=20 Chronically ART-supressed	2.7_{fold} increased transcription, no change in proviral HIV-1 DNA	Elliott et al 2014 [247]
	Single-arm, 3days/week dose for 8weeks, n=5 Chronically ART-supressed	No change in transcription nor proviral HIV-1 DNA	Archin et al 2014 [248]
Panobinostat (400mg)	Single-arm, 3days/week dose for 8 weeks, n=5 Chronically ART-supressed	2.9_{fold} increased transcription, no change in proviral HIV-1 DNA	Rasmussen et al 2014 [249]
Romidepsin ($5mg/m^2$)	Single-arm, 1dose/week for 3 weeks, (n=6) Chronically ART-supressed	3.8_{fold} increased transcription, detection of plasma VL, no change in proviral HIV-1 DNA	Søgaard et al 2015 [250]
Disulfiram (500mg) (500/1000/ 2000mg)	Single-arm, 14 daily infusion, n=16 Chronically ART-supressed	Significant increase by single copy assay	Spivak et al 2014 [251]
	3 doses arms, daily for 3 days	$1.7\text{-}2.1_{fold}$ increased transcription, increase by single copy assay	Elliott et al 2015 [252]
Bryostatin $10\text{-}20\mu g/m^2$	Single-dose placebo-controlled and 2 doses arm	No changes in HIV-1 transcription nor in plasma viral load	Gutierrez et al 2016 [253]
Interleukin-7 20mg/kg	Randomized placebo-controlled, n=29, Chronically ART-supressed	Increased plasma detection, significantly increased proviral HIV DNA	Katlama et al 2016 [254]

Table 4: Clinical trials of latency-reversing agents. Adapted from Rasmussend and Søgaard, Clinical Interventions in HIV Cure Research, 2018 [255]

Vorinostat was the first HDACi tested in chronically ARV-suppressed HIV-1 individuals, and it was shown to be able to increase HIV transcription *in vivo* by almost 5 fold, demonstrating for the first time that HIV latency could be disrupted [246]. However, testing alternative vorinostat dosing regimen (three doses per week for 8 weeks) was unable to increase viral transcription, and daily doses during 14 days reached lower levels of viral transcription than in the first study where it was used as a single dose [247,248]. Panobinostat was the second HDACi tested in a clinical trial and, when given 3 times per week for 8 weeks, showed an increased detection rate of plasma HIV RNA (non-quantitative) aside from an increment on HIV transcription (CA-US HIV RNA) but no changes in proviral HIV-1 DNA were observed [249]. At last, the HDACi that has proven to have a greater effect on reversing HIV latency is romidepsin. A pilot study with 3 weekly infusions of romidepsin (5mg/m^2), showed an increase in plasma HIV RNA quantifiable with a standard assay and demonstrated significant increases in HIV transcription (CA-US HIV RNA). Although these results demonstrate that it is possible to disrupt HIV latency using HDACi as LRAs, given alone, there is still no evidence of an effect in reducing HIV reservoir by their sole use *in vivo*. In addition, it is unclear whether RNA production levels achieved by treatment with HDAC are sufficient to sensitize a significant proportion of reservoir cells for CTL mediated elimination [256].

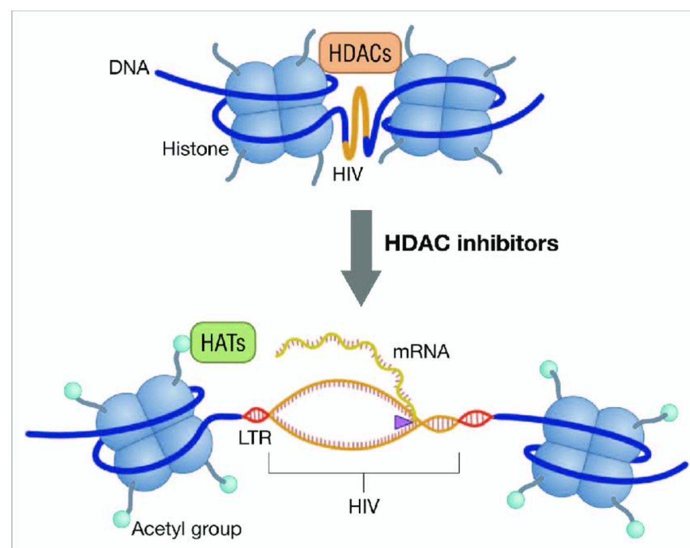

Figure 15: Disruption of HIV latency by HDAC inhibitors. Image from Rasmussen et al, Human Vaccines and Immunotherapeutics 2013 [257].

Other LRAs apart from HDACi have been tested in clinical trials. For instance, disulfiram, which acts on the PI3K/Akt pathway, was reported to activate HIV transcription *in vitro* [258]. When tested *in vivo*, disulfiram treatment was able to increase levels of HIV RNA quantifiable only when used at high dose and did not show any effect on HIV reservoir [252]. The PKC agonist bryostatin was also tested in a clinical trial but had no effect on the transcription of latent HIV [253]. A randomized-placebo controlled trial that tested ART intensification along with interleukin-7 (IL-7) administration was able to reactivate HIV but overall resulted in an increased reservoir size as it induced proliferation of the latently infected cells as a result of the homeostatic effect of this cytokine [254]. Other LRAs such as TLR7 and TLR9 have been tested in SIV, showing increases in plasma SIV viral particles and viral RNA, along with an increase of activated cytotoxic NK cells and $CD8^+$ T cells [259]. TLR9 has already been tested in a phase I clinical trial, leading to an improved innate immunity upon treatment, as well as HIV-1-specific adaptive immunity in HIV-1+ individuals [260]. Phase II clinical trials in HIV-individuals are now being performed to evaluate the effects of the TLR agonists given alone, such as vesatolimod (formerly GS-9629), a TLR-7 agonist being tested in a phase II clinical trial (NCT02858401), or in combination with other immunotherapies such as broadly neutralizing antibodies 3BNC117 and 10-1074 along with the TLR9 agonist lefitolimob that will be tested in the TITAN study (NCT03837756).

From the clinical trials mentioned and summarized in table 3, the one common conclusion is that LRAs are likely not able to decrease the frequency of latently infected cells if used alone. Although the regimens and the dose concentrations of the LRAs need to be further evaluated in order to find the best drug with the best kick&kill strategy design, there is a concern towards the killing part. While it was first thought that reactivation of the virus itself would lead to immune activation; it is now believed that further stimulation of the immune effector response will be needed in order to completely eliminate the reactivated infected cells. For this reason, combination of immunotherapies with LRAs are being investigated. The combinations tested so far include LRA mostly with T cell vaccines that aim to activate the adaptive arm of the anti-viral immune response. A list of completed clinical trials is shown in Table 5.

Trial	T cell vaccine + LRA	Trial design and size	Effect	References
RISVAC03 (NCT01571466)	MVA-B + disulfiram (250 mg)	4 vaccines every 4 weeks followed by 3 months of daily doses of disulfiram (n=12) ATI up 8 weeks	No changes in HIV DNA proviral copy numbers Modest delay in viral rebound in vaccinated individuals but not with disulfiram	Mothe et al 2015 [261]
REDUC (NCT02092116)	Vacc-4x + Romidepsin ($5mg/m^2$)	6 weekly vaccines followed by 3 weekly doses of romidepsin (n=20) MAP up to 16 weeks	3.1_{fold} increase in HIV transcription 40% decrease in proviral DNA copy numbers No delay on viral rebound	Leth et al 2016 [262]
RIVER (NCT02336074)	Chad.HIVconsv /MVA.HIVconsv + vorinostat	ChAd prime, MVA boost after 8 weeks, 10 doses of vorinostat every 3 days (n=52), no MAP	No decrease in HIV DNA proviral copy numbers No effect on the reservoir	AIDS 2018, Abstract TUAA0202LB IAS 2018
BCN02-Romi (NCT02616874)	MVA.HIVconsv/ + romidepsin ($5mg/m^2$)	MVA prime, followed by 3 weekly infusions of romidepsin, and MVA boost (n=15) MAP up to 32 weeks	Increased HIV transcription No significant changes in proviral DNA copy numbers Viral control in 23% of individuals up to 32 weeks of MAP	CROI 2017, Abstract 119LB, Mothe,Rosas et al 2019 (submitted)

Table 5: Clinical trials combining LRA with immunotherapies of latency-reversing agents.

The first kick&kill study performed, RISVAC03, used the therapeutic vaccine MVA-B, which contained the codon-optimized HIV-1 clade B inserts BX08-gp120 and IIIB-Gag-Pol-Nef (GPN), and daily doses of disulfiram for 3 months. Although a modest but statistically significant delayed viral rebound was observed in vaccinees, there was no effect on the proviral reservoir despite disulfiram treatment [261]. Subsequently, the REDUC study tested the peptide-based vaccine Vacc-4x expressing 4 Gagp24 peptides (Bionor Pharma), with rhGM-CSF as local adjuvant and three weekly doses of romidepsin. This combined intervention showed an increased HIV transcription and a modest decrease in latently $CD4^+$ infected cells measured as HIV DNA [262]. However, there was no effect on viral control when cART treatment was interrupted. In the RIVER trial, a randomized, open label trial including 60 HIV-1 positive individuals that had started cART during acute/recent HIV-1 infection compared the effect on the viral reservoir of the administration of 10 doses of vorinostat given after a vaccine regimen consisting of ChAdv.HIVconsv prime and MVA.HIVconsv boost. In this study, no further decrease in the number of HIV-1 DNA copies in PBMC $CD4^+$ T cells was observed in the vaccine and vorinostat arm although the intervention was able to boost functional $CD8^+$ and $CD4^+$ HIV-specific T cells and maintain these responses over time at a higher levels compared to the individuals in the cART alone arm (AIDS 2018, Abstract TUAA0202LB).

The only kick&kill clinical trial that has shown some signal of potential post-intervention control is the BCN02-Romi trial which will be discussed in detail in this thesis. HIV-infected individuals treated within less than 6 months after estimated HIV-1 acquisition, who had previously received a prime/boost vaccination regimen with ChAd63/MVA.HIVconsv in the parental BCN01 trial [96] were rolled-over into BCN02-Romi after they had reached 3 years under optimal ARV suppression. During BCN02, participants received two additional MVA.HIVconsv booster vaccinations in combination with three weekly doses of romidepsin before entering a monitored antiretroviral pause for a maximum of 32 weeks. Remarkably, 23% of the individuals showed sustained suppressed levels of viremia after the treatment interruption phase. Although BCN02 was an open label, single arm, proof-of-concept study, it is the first time that an immune-mediated viral control has been observed after a kick&kill strategy.

Apart from the completed studies summarized in table 4, there are several kick&kill ongoing clinical trials using different combinations. For instance, HIVACAR (NCT03619278) will use a combination of a personalized HIV RNA vaccine,

romidepsin and the broadly neutralizing antibody 10-1074. The HDACi panobinostat is being used in combination with pegylated interferon-alpha 2a therapy (NCT02471430), a therapeutic agent proposed to decrease levels of cell associated HIV-1-DNA [263]. Pegylated interferon-alpha 2a is also being tested along with a dendritic cell-based vaccine pulsed with autologous inactivated HIV (NCT02767193) and with the bNAbs 3BNC117 and 10-1074 (NCT03588715). The other HDACi used so far, vorinostat, is being used in combination with the HIV 1 antigen expanded specific T cell therapy (HXTC) (NCT03212989) and in combination with the antibody VRC07-523LS (NCT03803605). LRAs are also being used in combination with other strategies apart from T cell vaccine, such as the use of LRA with antibodies in ROADMAP and eCLEAR studies which combines romidepsin and the antibody 3BNC117 (NCT02850016, NCT03041012) or in the TITAN study (NCT033837757) that combines the TLR9 agonist lefitolimod with the antibodies 3BNC117 and 10-1074. Results coming from all those clinical trials will help define the most promising compounds or combinations that can induce both, HIV reactivation and elimination of latently infected cells.

3.6 Correlates of protection in HIV cure strategies. The need for treatment interruptions

To date, no robust, functional immune correlates have been identified that could serve as reliable predictors of viral control after curative interventions [264–266]. Identifying virologic and/or immunological biomarkers that correlate with the time to viral rebound or the ability to maintain viral suppression to low levels without cART could greatly accelerate and improve the evaluation of HIV cure strategies. So far, only levels of proviral DNA before any intervention have been associated with time to viral rebound after treatment interruption [267–270]. Therefore, the evaluation of new HIV cure strategies requires the demonstration of efficacy through antiretroviral treatment interruption (ATI) [265]. Nevertheless, stopping cART treatment has raised concerns about infected individuals' safety, selection of drug resistant HIV variants, reseeding the reservoir and the risk for HIV transmission. Although cART interruptions were performed in the past to diminish drug-associated toxicity, one large treatment interruption trail, known as the SMART, demonstrated a significantly increased risk of opportunistic diseases, cardiovascular and other non-AIDS adverse events in individuals on intermittent ART [268]. Since then, ATI are only recommended in the setting of clinical trials in which an efficacy endpoint is assessed. Given the potential

risks for ATI for the infected individual as well as for its sexual partners, clinical trials with ATI should also exclude patients with low CD4 counts, limit the duration of treatment cessation and use close monitoring and stringent immune and virological criteria for treatment resumption. Some past therapeutic vaccine trials including ATI used control of viremia and/or prevention of $CD4^+$ T cell decay as the primary trial endpoint [223,271,272]. Later on, a "monitored anti-retroviral pause" (MAP) became more frequently used in clinical trials were the primary endpoint was the time to viral rebound, considering that re-starting treatment at a pre-set low viral load was potentially less harmful than at a pre-defined period off treatment [273]. A recent metanalysis of 22 studies including treatment interruption concluded that treatment cessation is a safe strategy to use in clinical trials when a close monitoring for HIV-1 rebound is accomplished. In addition, the description of the PTC phenotype has moved the field towards longer monitored interventions where a period of viremia is needed before control is achieved. Recommendations for analytical antiretroviral treatment interruptions in HIV research trials have been developed in order to coordinate a consensus for treatment interruption and the interpretation of study results [274].

As shown in Figure 15, while viral rebound without any intervention takes place at approximately 2 weeks after treatment interruption, non-CCR5-Δ32 stem cell transplant resulted in a 3 to 10 months delayed rebound. Other cases mentioned earlier like the Mississippi baby or the VISCONTI cohort resulted in 2 to 12 years of viral remission. Only the "Berlin patient" has been without viral rebound for more than 12 years now. Overall, better predictors of viral rebound during treatment interruption are urgently needed to reduce the risk of conducting unnecessary treatment interruptions and to adjust trial design to maximize vaccination outcome. Of note, immune correlates of control would also help improve the design of novel preventive approaches to avoid HIV infection.

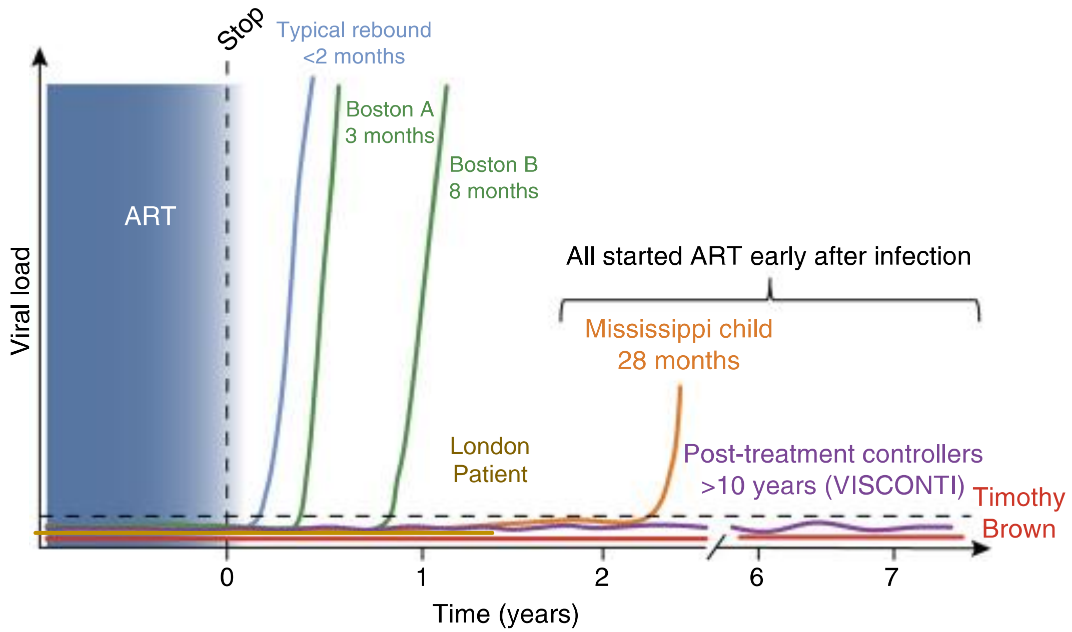

Figure 16: Time in remission and viral rebound after ART cessation in HIV cure trials. Image modified from Deeks et al, Nature Medicine 2016 [275].

In the following pages, detailing the work conducted in this thesis, we aim to provide a better understanding of the mechanisms of HIV immune control as well as to identify potential correlates of immune-driven HIV-1 control in intervention clinical studies after an antiretroviral treatment interruption.

HYPOTHESIS AND AIMS

Despite the fact that current combination ART is highly effective in suppressing HIV-1 replication to undetectable levels, HIV-1 remains present in the body due to the viral reservoir that is established during the initial stages of the infection. Consequently, treatment interruption results in a rapid viral rebound within weeks in nearly all individuals. Many strategies are being investigated to prevent such a viral rebound and in order to achieve either a functional cure of HIV-1 infected individuals (similar to EC phenotype) or, even, a sterilizing cure (complete elimination of the virus). Some of these strategies include early treatment initiation and cART intensification, passive transfer of antibodies, therapeutic vaccination, kick and kill strategies, stem cell transplantation and gene therapy

The hypothesis of this thesis is that an immune-driven cure strategy may achieve a functional cure of HIV-1 infection by enhancing the virus-specific, cellular immune response able to control viral reservoir and eliminate HIV-infected cells.

The global aim of this work is to identify immune correlates of relative HIV control and translate this knowledge into assessing and improving the efficacy of future kick and kill strategy.

The specific aims are:

Chapter I

Identify virological and immunological parameters involved in abrupt transition to progressive HIV infection in a cohort of previous HIV controllers.

I. Determine the viral evolution preceding abrupt loss of viral control, assessing viral tropism, identifying HIV-1 superinfections and evaluating the number of CTL escape mutations before and after loss of viral control.

II. Characterization of immune responses before and after losing HIV control including HLA typing and characterization of HIV-specific $CD8^+$ T cell responses and measuring its antiviral capacity.

Chapter II

Integrate host and vaccine-induced virological and immunological parameters to define potential predictors of viral outcomes after a structured treatment interruption in a therapeutic vaccine trial.

I. Examine changes in HIV-1 specific immune responses and HIV-1 proviral reservoir after treatment interruption.

II. Study the vaccine-exerted effects on the rebounding plasma virus population present after structured treatment interruption.

III. Define correlates of viral rebound dynamics after treatment interruption.

Chapter III

Evaluate the safety, immunogenicity and effect on the viral reservoir of the kick&kill strategy tested in the BCN02 clinical trial combining HIVconsv therapeutic vaccination with the LRA romidepsin in a cohort of early treated HIV^+ individuals.

I. Evaluate the safety and tolerability of MVA.HIVconsv vaccinations, romidepsin administration and monitored antiretroviral pause.

II. Measure the pharmacokinetics and pharmacodynamics of romidepsin in terms of HIV reactivation capacity and effects on the HIV reservoir.

III. Asses the immunogenicity of the MVA.HIVconsv vaccinations.

IV. Determine the factors involved in viral rebound kinetics after a monitored antiretroviral pause.

Chapter IV

Evaluate the *in vivo* effects of the histone deacetylase inhibitor romidepsin on T cell populations and functionality of vaccine-induced T cells responses in BCN02 clinical trial participants.

I. Assess the impact of romidepsin on T cell viability, memory phenotype populations and activation during the three romidepsin infusions.

II. Examine the functionality and antiviral capacity of HIV-specific T cells upon MVA.HIVconsv vaccination and after romidepsin treatment.

RESULTS

CHAPTER I

Mechanisms of abrupt loss of virus control in a cohort of previous HIV controllers

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Journal of Virology. 2019 Feb 5;93(4).

Abstract

Elite and viremic HIV controllers are able to control their HIV infection and maintain undetectable or low-level viremia in the absence of antiretroviral treatment. Despite extensive studies, the immune factors responsible for such exclusive control remain poorly defined. We identified a cohort of 14 HIV controllers that suffered an abrupt loss of HIV control (LoC) to investigate possible mechanisms and virological and immunological events related to the sudden loss of control. The in-depth analysis of these subjects involved the study of cell tropism of circulating virus, evidence for HIV superinfection, cellular immune responses to HIV, as well as an examination of viral adaptation to host immunity by Gag sequencing. Our data demonstrate that a poor capacity of T cells to mediate *in vitro* viral suppression, even in the context of protective HLA alleles, predicts a loss of viral control. In addition, the data suggest that inefficient viral control may be explained by an increase of CD8 T-cell activation and exhaustion before LoC. Furthermore, we detected a switch from C5- to X4-tropic viruses in 4 individuals after loss of control, suggesting that tropism shift might also contribute to disease progression in HIV controllers. The significantly reduced inhibition of *in vitro* viral replication and increased expression of activation and exhaustion markers preceding the abrupt loss of viral control may help identify untreated HIV controllers that are at risk of losing control and may offer a useful tool for monitoring individuals during treatment interruption phases in therapeutic vaccine trials.

Importance

A few individuals can control HIV infection without the need for antiretroviral treatment and are referred to as HIV controllers. We have studied HIV controllers who suddenly lose this ability and present with high *in vivo* viral replication and decays in their $CD4^+$ T-cell counts to identify potential immune and virological factors that were responsible for initial virus control. We identify *in vitro*-determined reductions in the ability of CD8 T cells to suppress viral control and the presence of PD-1-expressing $CD8^+$ T cells with a naive immune phenotype as potential predictors of *in vivo* loss of virus control. The findings could be important for the clinical management of HIV controller individuals, and it may offer an important tool to anticipate viral rebound in individuals in clinical studies that include combination antiretroviral therapy (cART) treatment interruptions and which, if not treated quickly, could pose a significant risk to the trial participants.

Material and methods

Study participants and samples.

Among two existing cohorts of HIV-1-infected elite and viremic controllers (defined by sustained undetectable or low-level viremia, respectively [plasma viral load {pVL}, <50 copies/ml or <2,000 copies/ml] for more than 1 year in the absence of ART), we identified 14 subjects who experienced an abrupt transition from a nonprogressive to a progressive state of HIV infection (Table 1). All individuals had stable viremia at <3 log copies/ml before LoC for a median time of 14 years since HIV diagnosis (interquartile range [IQR], 4 to 19 years). The transition to LoC was defined as (i) >1-log pVL increase and/or (ii) loss of >30% $CD4^+$ T-cell counts or drop below 350 CD4+ T cells/ml within 1 year. Samples were obtained from the Spanish HIVHGM BioBank supported by the Spanish Instituto de Salud Carlos III and from the existing long-term sample repository at IrsiCaixa, in Badalona, Spain. All subjects were HLA-typed for HLA-A, HLA-B, and HLA-C loci at high resolution, as described previously [31]. Data from a full-protein ELISPOT CTL screen of a group of 65 individuals from the IrsiCaixa Controllers Cohort with persistent HIV control for a median time of 14 years since HIV diagnosis (IQR, 5 to 21 years) were included for comparisons and included 38 EC (median viral load, 50 copies/ml; median CD4 count, 815 cells/mm^3) and 27 viremic controllers (median VL, 545 copies/ml; median CD4 count, 616 cells/mm^3). A subset of eight HIV-1 controllers with longer clinical follow-up and with prospective biological samples available at separated time points was selected for the viral inhibition assay comparison as long-term controllers (Table S1). All patients provided informed consent before providing samples. The study was approved by the institutional ethical review board of the Hospital Universitari Germans Trias i Pujol (reference no. EO-09-041).

Gag sequence evolution

HIV-1 RNA was extracted from plasma using the QIAamp viral RNA kit (Qiagen) and was reverse transcribed using reverse transcription-PCR (RT-PCR) SuperScript III enzyme mix (Invitrogen). The gag gene region was then amplified by nested PCR. Sequencing was performed by the Genomics Core Facility at Germans Trias i Pujol Research Institute. Gag nucleotide alignment was performed with MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) to generate the corresponding multiple-sequence alignments (MSA). Nucleotide evolution models that best explained sequence evolution within each MSA were identified using the MEGA5 Find Best DNA/Protein Models (ML) function. The model with the lower score according to Bayesian information criteria implemented in MEGA5 was selected to construct phylogenetic trees. Gag sequences obtained were further analyzed to determine the number of HLA class I-

associated immune escape mutations and were identified based on the individual's HLA class I genotype and the HLA-associated escape mutations described by Brumme et al. [23].

Determination of viral tropism

HIV tropism was predicted by Gp160 V3-loop region Sanger sequencing, as previously described [63]. Briefly, HIV-1 RNA was extracted from plasma using the QIAamp viral RNA kit (Qiagen), and cDNA was synthetized using RT-PCR SuperScript III enzyme mix (Invitrogen). The env V3-loop-encoding region was amplified by nested PCR. Sequences were analyzed by using the Geno2Pheno [coreceptor] using a false-positive rate (FPR) cutoff of 10% to define the presence of an X4 HIV-1.

IFN-γ ELISPOT

ll individuals were screened longitudinally for IFN-γ-secreting T-cell responses to the entire HIV proteome. Briefly, cryopreserved PBMC were thawed and rested for 5 h at 37°C before plating 100,000 live cells per well in IFN-γ ELISPOT 96-well polyvinylidene plates (Millipore). PBMC were stimulated with a clade B consensus sequence set of 410 peptides (18mers overlapping by 11 amino acids [aa]) at a final concentration of 14 µg/ml. The IFN-γ Mabtech kit was used, according to the manufacturer's instructions. Spots were counted using an automated ELISPOT reader system (ImmunoSpot S6 Versa; CTL, Germany), and the magnitude of responses was expressed as spot-forming cells (SFC) per million input cells. The threshold for positive responses was defined as at least 5 spots per well, responses exceeding the "mean number of spots in negative-control wells plus 3 standard deviations of the negative-control wells" and "three times the mean of negative-control wells," whichever was higher.

Viral inhibition assay

The capacity of $CD8^+$ T cells to suppress HIV-1 replication in autologous $CD4^+$ T cells *ex vivo* was assessed as described in detail elsewhere [69,70]. Briefly, CD8-depleted cells were isolated from cryopreserved PBMC by magnetic bead separation (MACS Milteny Biotec) and stimulated with phytohemagglutinin (PHA; 5 µg/ml) for 3 days in R10-RPMI medium supplemented with 10% fetal calf serum (FCS), l-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were then washed and infected by spinoculation with an HIV-1 IIIB strain at a multiplicity of infection (MOI) of 0.01. To assess the antiviral capacity of $CD8^+$ T cells, autologous infected $CD4^+$ T cells were cultured alone or with unstimulated $CD8^+$ T cells in R10 supplemented with interleukin 2 (20 IU/ml). The experiments were carried out in triplicate at different effector-to-target ratios (1:1, 1:10, and 1:2, depending on cell availability). After 6 days of coculture,

intracellular p24 production was measured by flow cytometry. Antiviral capacity was expressed as percentage of inhibition, determined as [(fraction of p24$^+$ cells in CD4$^+$ T cells cultured alone) − (fraction of p24$^+$in CD4$^+$ T cells cultured with CD8$^+$ cells)]/(fraction of p24$^+$ cells in CD4$^+$ T cells cultured alone) × 100.

Flow cytometry

CD8$^+$-depleted cells (CD4$^+$ T-cell enriched fraction) and CD8$^+$-isolated T cells were stained for activation and exhaustion markers. Cells were first stained with a viability staining (Aqua LIVE/DEAD fixable dead cell stain kit; Invitrogen), followed by exclusion staining of B lymphocytes and myeloid cells using combined markers in a dump channel (using the cells/antibodies CD19/BV510 and CD14/BV510; BioLegend). For T-cell lineage and activation markers, the following cells/antibodies were used: CD3/antigen-presenting cells (APC) Cy7, CD4/peridinin chlorophyll protein (PerCP) (BD Biosciences); and CD8/PerCP, CCR7/BV421, CD45RAPECF594/HLA-DR fluorescein isothiocyanate (FITC), PD-1/phycoerythyrin (PE,) CD69/APC, CD38/BV785, and CD25/BV605 (BioLegend). After staining, cells were collected on an LSRFortessa instrument (BD), and analysis was performed using the FlowJo 10 software.

To determine the number of p24$^+$ cells in the viral inhibition assay, cells were stained with Aqua LIVE/DEAD (Invitrogen), followed by extracellular staining with cells/antibodies CD3/APC-H7, CD4/PerCP, and CD8/APC (BD Biosciences). Cells were fixed and permeabilized (FIX & PERM cell fixation & cell permeabilization kit; Thermo Fisher Scientific) and finally stained with p24 antibody KC.57 fluorescein isothiocyanate (FITC; Beckman Coulter). Samples were acquired on an LSR-II cytometer, and data analysis was done using the FlowJo 10 software.

Statistical analysis

GraphPad Prism version 7.0 for Windows (San Diego, CA) was used to compare response rates in both groups and subgroup analyses. A Mann-Whitney test and Wilcoxon matched paired test were used for unpaired and paired comparisons, respectively.

Data availability

Gag sequences are available at GenBank with the accession numbers MK086511 (LP1), MK086512 (LP2 pre), MK086513 (LP2 post), MK086514 (LP3 pre), MK086515 (LP3 post), MK086516 (LP4 pre), MK086517 (LP4 post), MK086518 (LP5 pre), MK086519 (LP5 post), MK086520 (LP6 post), MK086521 (LP7 post), MK086522 (LP8 pre), MK086523 (LP8 post), MK086524 (LP9 pre), MK086525 (LP9 post), MK086526 (LP10

post), MK086527 (LP11 pre), MK086528 (LP11 post), MK086529 (LP12 pre), MK086530 (LP12 post), MK086531 (LP13 post), MK086532 (LP14 pre), and MK086533 (LP14 post)

Introduction

There is a small proportion of HIV-1-infected individuals that spontaneously control HIV infection [1,2]. Due to the heterogeneity among individuals with this clinical course [3,4] they are referred to as long-term nonprogressors (LTNP), HIV controllers, or, in the case of undetectable viremia, elite controllers. Several factors have been postulated to play a role in this viral control, including host genetic, immunological, and viral factors. In particular, host genetic markers have been associated with disease progression, yet their mechanistic action remains uncertain [5]. Possibly, the strongest predictors of HIV control include polymorphisms in HLA class I alleles, which alone or in combination with killer cell immunoglobulin-like receptors (KIR) have been linked to sustained low-level viremia in the absence of combination antiretroviral therapy (cART) [6-8]. Since the HLA class I-encoded gene products present virus-derived T-cell epitopes to $CD8^+$ T cells, an extensive number of studies exist that have also linked T-cell responses and their specificities to HIV control [2,9,10]. Aside from host genetics and immune factors, viral factors, such as viral replication capacity and cell tropism, have been associated with HIV control, although cell tropism has not been consistently documented [11,12].

During the course of HIV infection, a proportion of nonprogressor individuals may suffer a disruption of their capacity to control infection, which can manifest itself in different ways, as follows: clinical progression defined as a new AIDS-defining event, immunological progression defined as an abrupt decrease of $CD4^+$ T-cell counts, and/or virological progression as a significant increase in viral loads [13-16]. In addition, HIV superinfection has been identified as a possible explanation for sudden signs of uncontrolled HIV infection [17]. Specific plasma cytokine profiles and Gag-specific T-cell responses have been linked as well to eventual loss of control in an elite controller cohort [18]. However, the contribution of these factors to a sudden loss of control is poorly defined, in part due to the scarce availability of longitudinal samples from such individuals, especially of samples close to the time point before loss of viral control (LoC). Here, we have identified and longitudinally followed 14 individuals who experienced an abrupt transition to progressive HIV infection with pre- and post-LoC samples available within 1 year, and we have integrated host, virological, and immune parameters in order to better define the mechanisms underlying the progression of HIV-1 infection.

Results

Clinical characteristics of HIV controllers experiencing abrupt LoC.

For the present analyses, 14 individuals with LoC were identified after reviewing clinical criteria and sample availability for HIV-infected individuals with elite controller (EC) or viremic controller (VC) status from the IrsiCaixa and the BioBank Controllers cohorts (Table 1). The evolution of viral load and CD4 counts is shown in Fig. S1 in the supplemental material. Samples prior to LoC were obtained with a median of 20 months before the first signs of LoC defined either as detection of uncontrolled viral load ($n = 4$) or a concomitant raise in viral load and reduction in CD4 counts ($n = 10$, Fig. S1). Follow-up samples included samples taken at first diagnosis of LoC ($n = 11$) or within the following 6 months ($n = 3$). As HIV control has been linked to HLA class I genotypes, high-resolution HLA class I typing was performed on all 14 individuals included in the study. In line with data from other cohorts of elite and viremic controllers [19,20], protective HLA class I alleles B57 and B58 were more common in this group of individuals (Fig. 1) than in the general population in Spain and similar to the frequencies seen in the EC and VC comparison group established at our center. Thus, in this relatively small cohort, no specific class I HLA was associated with LoC in individuals with previous HIV control.

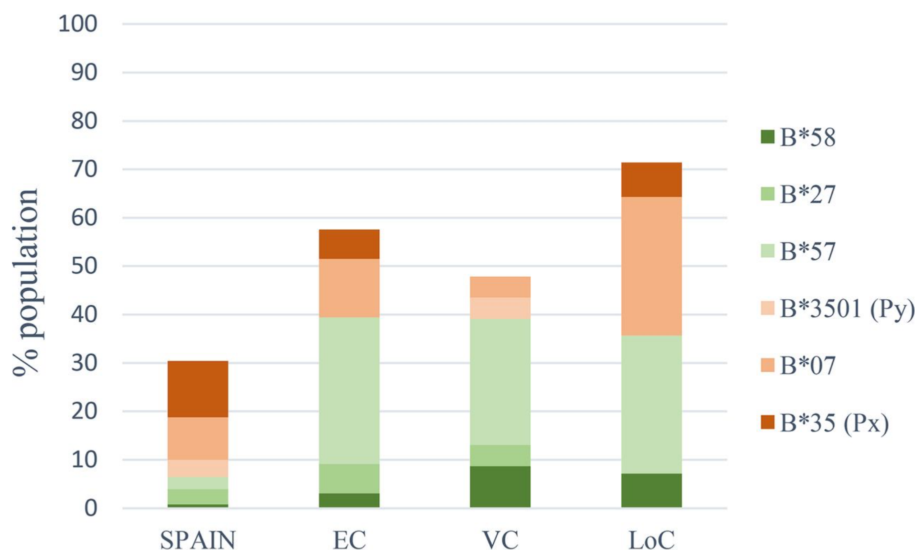


Figure 1. HLA class I allele frequencies. HLA-B allele frequencies are shown for the general Spanish population [64], a cohort of elite controllers (EC, n = 38), viremic controllers (VC, n = 27), and individuals that have abruptly lost their capacity to control the infection (LoC, n = 14).

Table 1. Clinical characteristics of the 14 controllers with loss of HIV control

ID[a]	Age (yr)[b]	Sex	Time since known HIV-1 infection (yr)[b]	Log_{10} of viral load at control timepoint studied[c]	Log_{10} of viral load at peak of loss of control	No. (%) of CD4 cells/mm³ at control time point studied	No. (%) of CD4 cells/mm³ at loss of control	HLA-A[e]	HLA-B[e]	HLA-C[e]	HLA-DRB[e]	HLA-DQB[d,e]
LP1	44	Male	16	UD	3.66	1,007 (37)	426 (18)	0201/3002	1801/5701	0501/0602	0301/0701	0201/0303
LP2	54	Female	25	3.13	5.18	721 (44)	171 (19)	0201/3402	1401/4402	0501/0802	1301/1454	NA
LP3	42	Male	3	2.74	4.36	507 (26)	265 (17)	0101/1101	3508/5201	0401/1202	0801/1502	0402/0601
LP4	23	Male	3	UD	4.90	1148 (32)	858 (29)	0201/2402	1501/4403	0303/1502	0103/1103	0301/0501
LP5	37	Male	13	2.84	5.63	495 (25)	266 (15)	0201/0301	5101/5101	1502/1502	1301/1301	1603/1603
LP6	47	Female	25	UD	3.70	438 (25)	282 (18)	0201/2902	4001/5701	0304/0602	0401/0701	0302/0303
LP7	36	Male	4	2.40	4.72	1,128 (47)	555 (31)	0101/3101	4001/5701	0304/0602	0404/0701	0302/0303
LP8	43	Female	18	3.49	5.20	534 (33)	166 (11)	0301/2501	0702/5101	0702/0303	0901/1101	0301/0303
LP9	44	Male	14	3.66	5.02	748 (21)	402 (22)	0301/6802	0702/5301	0702/0401	1301/1302	0603/0604
LP10	38	Male	13	UD	5.90	913 (32)	400 (21)	1101/2402	0702/5101	0702/1502	0803/1501	0301/0602
LP11	44	Male	14	2.96	5.36	920	464	0201/3001	4501/5701	0602/0701	0405/1502	0302/0601
LP12	45	Male	23	2.93	4.00	810	493	0201/3201	1302/1501	0303/0602	0701/1401	0202/0301
LP13	45	Male	16	2.52	3.64	434 (32)	266 (22)	0301/1101	0702/5201	0702/1202	1301/1502	0601/0603
LP14	35	Male	1	2.63	5.20	430	465	0205/2402	0801/5801	0701/0702	0301/1102	0201/0301

[a]ID, identifier; LP, late progressor.
[b]Age and HIV duration are shown for the latest time point analyzed.
[c]UD, undetermined.
[d]NA, not available.
[e]Individual HLA class I typing is shown (A, B, C, DRB, and DQB).

Evidence for virus tropism change and superinfection between pre- and post-LoC time points.

In order to determine whether changes in the cellular tropism of plasma virus preceded the abrupt loss of virus control, we determined viral tropism in plasma samples drawn before (n = 9; median, 21 months; range, 5 to 74 months) and after (n = 11), at diagnosis (n = 8), or during the peak of viremia (n = 3) clinical progression. Of the 14 samples obtained while the individuals had controlled infection, viral sequences covering the V3-loop (gp160) in all cases were indicative of a dominant CCR5-tropic viral population. In contrast, CXCR4-tropic plasma viruses were detected in four individuals post-LoC (although one of the individual was approaching the false-positive threshold of 10%), of which three had confirmed R5 virus pre-LoC (one did not amplify for env; Table 2). Based on phylogenetic analyses of Gag sequences, all pre- and post-LoC sequences showed close clustering for each individual except for one (subject LP4, Fig. 2), suggesting a superinfection event. Overall, clear evidence for changes in cell tropism were observed in one-quarter of the studied cohort, while superinfection was not a generalized mechanism for LoC in this group of patients.

Table 2. HIV tropism before and after loss of control HIV tropism was estimated by Gp120 V3-loop region sequencing before and after late progression

ID	Control-Pre (%FPR)	LoC-Post (%FPR)
LP1	NA	CCR5 (12.5)
LP2	CCR5 (36.2)	CXCR4 (0.1)
LP3	CCR5 (35.5)	CCR5 (35.3)
LP4	NA	CXCR4 (3.2)
LP5	CCR5 (13.2)	CXCR4 (8.6)
LP6	NA	NA
LP7	CCR5 (36.2)	CXCR4 (4.8)
LP8	CCR5 (10)	CCR5 (41.4)
LP9	CCR5 (55)	CCR5 (28.8)
LP10	CCR5 (42.3)	CCR5 (28.8)
LP11	CCR5 (38)	CCR5 (37.4)
LP12	NA	CCR5 (86.5)
LP13	NA	CCR5 (42.2)
LP14	CCR5 (55.1)	CCR5 (55.1)

[a]The false-positive rate (FPR) was set to 10% to identify X4 tropism. NA, sample not amplified.

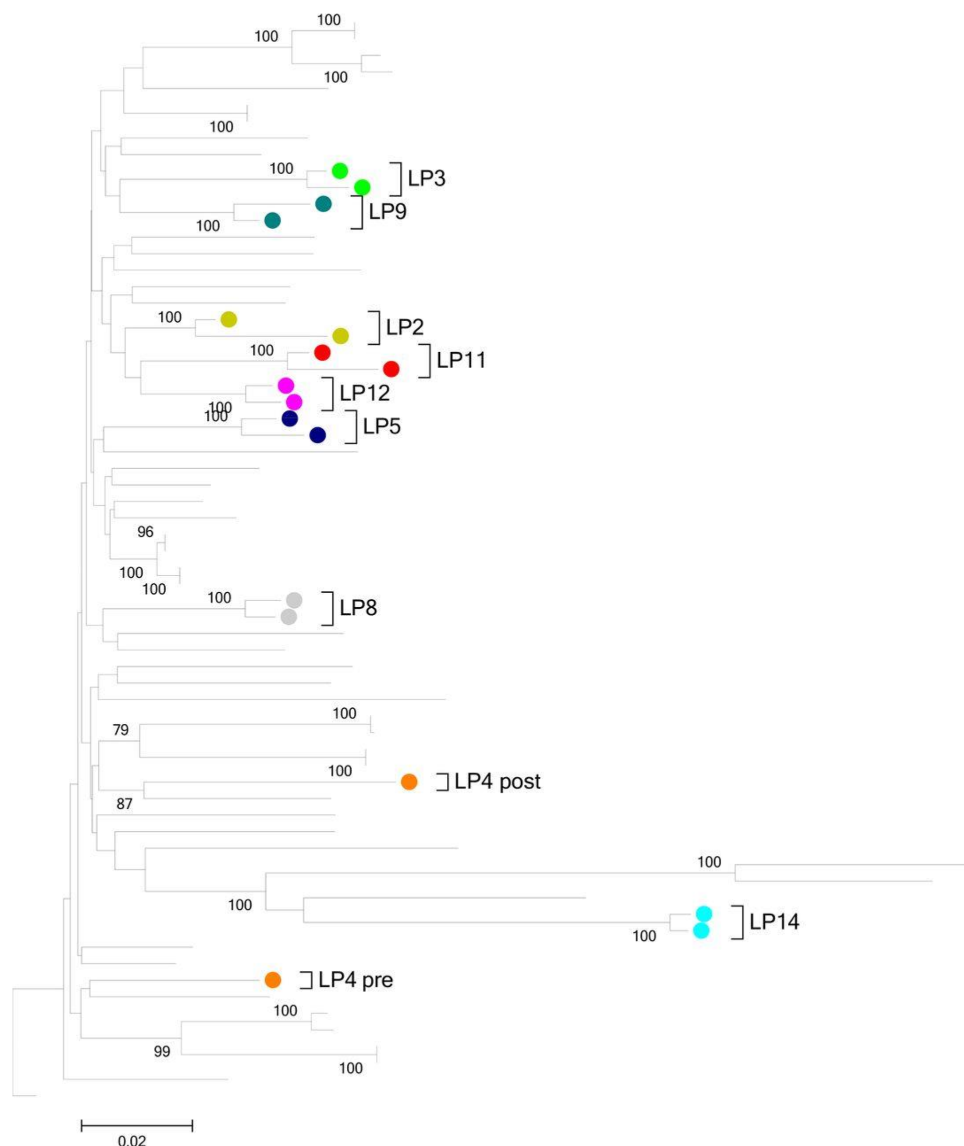


Figure 2. Evolutionary relationships of taxa. The evolutionary history was inferred using the neighbor-joining method [65]. The optimal tree with the sum of branch length of 1.91362276 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches [66]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method [67] and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis was based on a 66-nucleotide amplicon in the V3-loop of HIV gp120. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA5 [68].

Virus maintained HLA-associated escape mutations.

Viral breakthrough from immune surveillance has been described for both T- and B-cell-mediated immune control and could contribute to the sudden raise in viral loads and clinical HIV disease progression [21, 22]. In order to evaluate whether the presence and accumulation of T-cell escape mutations contribute to LoC, HIV Gag sequences were

obtained from samples drawn before and after LoC and analyzed for the presence of HLA class I footprints [23] (Fig. 2). All individuals yielded sequences in post-LoC samples, while HIV Gag sequences were successfully amplified in 9 out of 14 pre-LoC samples when subjects presented with effective *in vivo* virus control and low or undetectable viral loads. Gag sequences were compared with described HLA-associated escape mutations specific to the individuals' HLA class I genotype [23]. In 67% of patients with pre- and post-LoC sequences available, the number of patient-HLA specific footprints increased from pre- to post-LoC sequences. Despite this trend, the median number of footprints post-LoC (median, 3 footprints; range 1 to 8 footprints) did not differ significantly from the frequency of HLA footprints pre-LoC (median, 2 footprints; range, 1 to 5 footprints; Fig. 3, P = 0.2682). Although limited to Gag, the target of some of the most effective antiviral T-cell responses [10], and although single epitope mutations can lead to a complete loss of control, these data do not suggest that broad cytotoxic T lymphocyte (CTL) escape on multiple epitopes is the major driving force for LoC in the present cohort.

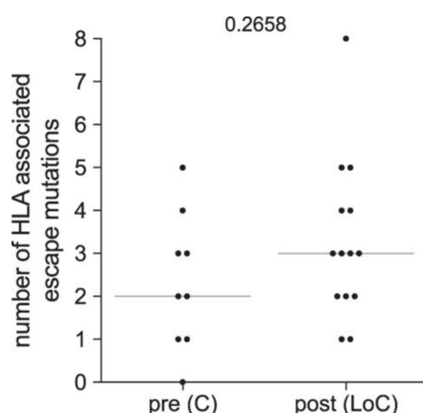


Figure 3. Number of HLA escape mutations in Gag [23] detected in viral population from samples before and after loss of control.

Increased number of HIV-specific T-cell responses post-LoC.

Individuals that control HIV infection have been shown to have a robust HIV-1-specific $CD8^+$ T-cell response, especially against the Gag protein, compared to responses observed in noncontrollers [10, 24]. The HIV-specific $CD8^+$ T cells responses before LoC were thus measured and compared to those of post-LoC samples and to responses seen in 65 individuals from the IrsiCaixa Controllers cohort. Responses to the entire HIV proteome were measured by gamma interferon (IFN-γ) enzyme-linked immunosorbent spot assay (ELISPOT) using a 410-overlapping-peptide set, covering the complete HIV genome, as described previously [25]. Twelve out of the 14 individuals had sufficient cell viability to conduct longitudinal full-proteome HIV ELISPOT screens (Fig. 3). In nine of them, the number of individual responses remained stable (n = 1 individual) or increased

(n = 8 individuals), with a significant increase in the median breadth of response from 6 responses (range, 1 to 14 responses) pre-LoC to a median of 11 responses (range, 2 to 20 responses; Fig. 4A; P = 0.0488). Even though the appearance of more responses post-LoC may be due to higher viral replication and antigen availability post-LoC, the increase in the breadth of responses did not result in a significant increase in the total magnitude (median magnitude from pre-LoC, 3,145 spot-forming cells [SFC]/million peripheral blood mononuclear cells [PBMC; range, 100 to 7,950 SFC/million PBMC] to median post-LoC of 4,745 SFC/million PBMC [range, 690 to 27,420 SFC/million PBMC; Fig. 4B; P = 0.6221]. Of note, the dominance of Gag-specific responses which characterizes naturally controlling individuals was not lost after LoC and was similar to levels tested in the comparison cohort of elite and viremic controllers (Fig. 4C andS3). Furthermore, there was no consistent pattern in the emergence of new and expansion/reduction of preexisting responses from pre- to post-LoC; however, while some individuals showed a clear loss of immunodominant responses to certain HLA-restricted epitopes in the absence of detectable mutations, other patients maintained a stable response pattern (Fig. S3). Furthermore, there were no differences in the changes in responses between individuals in whom the virus changed tropism and individuals that maintained R5-tropic virus. Interestingly, the one individual (LP4) that showed evidence of superinfection had a limited breadth of responses pre-LoC (4 responses), of which only 2 were still detectable post-LoC, while there were an additional 15 responses detected post-LoC, possibly reflecting new targets in the superinfecting virus. In this individual, viral sequence data for Gag indicated that the pre-LoC virus contained all described optimal epitopes for his HLA class I alleles in wild-type immunogenic sequence, yet the subjects only mounted these Gag responses upon uncontrolled superinfection [17].

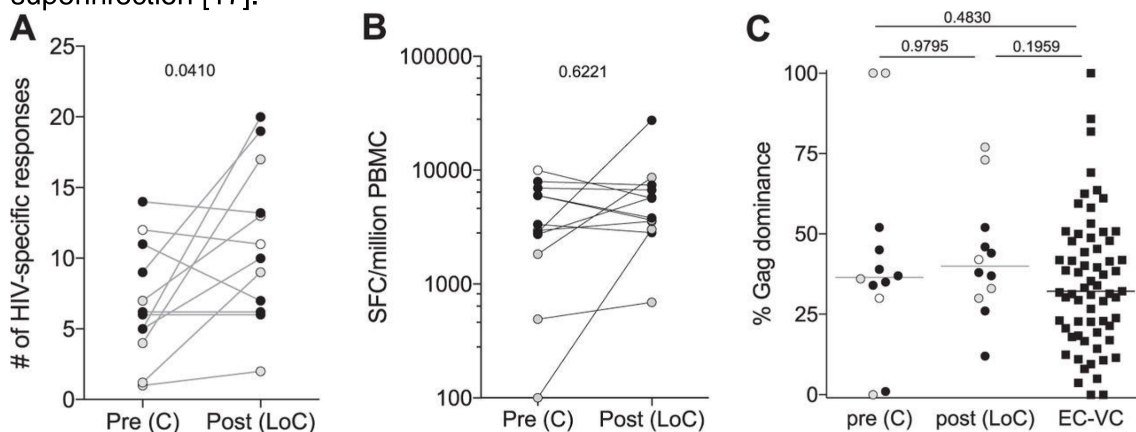


Figure 4. HIV-specific T-cell responses. (A) Breadth of HIV-specific T-cell responses before and after loss of control (n = 12). (B) Magnitude of responses, expressed as SFC/million PBMC, is shown longitudinally (C) Gag dominance (magnitude of Gag responses/total magnitude) is shown before and after evolution in

the 12 LoC subjects and compared to a cohort of elite (EC) and viremic (VC) controllers (n = 65). Individuals that maintained an CCR5 tropism are shown in black dots, individuals that changed to a CXCR4 are shown in gray dots, and the individual from which tropism was not available is shown in white dots. P values are shown for Wilcoxon signed rank test in a comparison of paired data and for Mann-Whitney test to compare between groups.

Reduced antiviral capacity of CD8$^+$ T cells after loss of virological control.

In vitro inhibition of viral replication by autologous CD8$^+$ T cells has become a standard measure to determine the *ex vivo* antiviral activity of cytotoxic T lymphocyte responses. We asked whether the viral inhibition assay (VIA) activity in individuals suffering an abrupt loss of control would be reduced at pre-LoC in comparison to persistent elite and viremic controllers and how VIA activity would change after LoC. In the absence of autologous virus isolates, the HIV IIIB laboratory strain was used to test *in vitro* inhibitory capacity of PBMC-derived CD8$^+$ T cells. As a comparison, isolated CD8$^+$ T cells from longitudinal PBMC samples of eight long-term HIV controllers with persistent *in vivo* HIV control (n = 6 EC and n = 2 VC, >15 years of diagnosed HIV-2 infection; longitudinal samples were 102 [range, 45 to 156] months apart between both follow-up time points) were tested (Table S1). Viral replication was measured by flow cytometry as a percentage of HIV Gag p24-positive cells in culture. The results showed a strong capacity of CD8$^+$ T cells from pre-LoC samples to inhibit HIV replication at a 1:1 E/T (effector-to-target cells) ratio. However, this activity at the 1:1 ratio among the LoC patients studied here was somewhat lower than that of HIV controllers (median, 65% of LoC individuals compared to 84% of controllers, P = 0.0650; Fig. 5A) and significantly weaker at the 1:10 E/T ratio (median, 23% of LoC compared to 50% of controllers, P = 0.0104; Fig. 5B). While virus-suppressive activity in long-term controllers was maintained over time (P = 0.8438; median, 102 months between both time points tested), the suppressive capacity in LoC further decreased, especially detectable at the 1:1 ratio (P = 0.0156, Fig. 5A) upon loss of control. Thus, the data suggest that declining VIA activity over pre-LoC time points may be a prognostic marker for an increased risk of LoC.

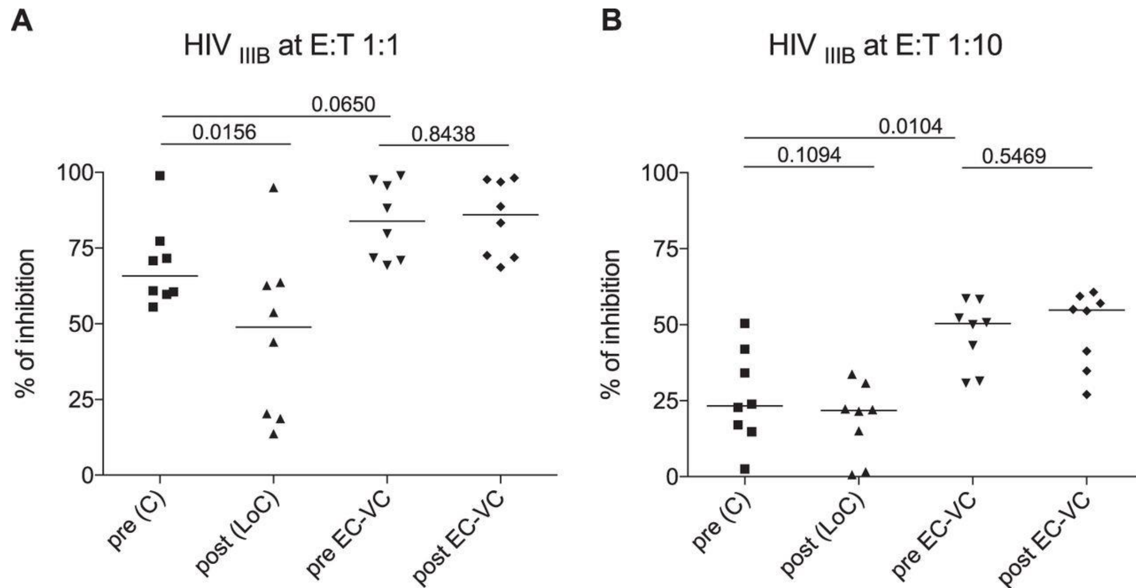


Figure 5. Viral inhibition capacity. Levels of CD8⁺ viral inhibitory capacity are shown for IIIB E/T 1:1 (A) and E/T 1:10 (B) in individuals who presented a loss of control (n = 8) and long-term controllers (n = 8). Comparison of antiviral capacity is expressed as % inhibition = [(fraction of p24⁺cells in CD4⁺ T cells cultured alone) − (fraction of p24⁺ cells in CD4⁺ T cells cultured with CD8⁺ T cells)]/(fraction of p24⁺ in CD4⁺ T cells cultured alone) × 100.

Elevated frequencies of CD38 and PD-1 expression in CD8⁺ T cells after LoC.

The expression of CD38 and HLA-DR on T cells has been shown to be lower in treated HIV-infected individuals under cART and in HIV controllers than in individuals with progressive HIV disease. We therefore asked whether individuals experiencing LoC had elevated levels of activated cells right before the loss of control and how the increased virus replication post-LoC may impact CD38 and HLA-DR expression. The expression of surface activation markers on CD4⁺ and CD8⁺ T cells was assessed by flow cytometry before and after a loss of HIV control, along with additional activation markers (HLA-DR, CD38, CD25, and CD69) and an exhaustion marker (PD-1). Longitudinal samples from persistent HIV controllers were tested for comparison (102 months apart; range, 45 to 156 months). The expression of activation markers pre-LoC was similar to that in long-term controllers (data not shown); therefore, there was no evidence for elevated residual viral replication in LoC that could drive higher levels of activated T cells. In contrast, an almost 2-fold increase in the percentage of CD38⁺ and a trend toward higher levels of HLA-DR⁺ CD8 cells were observed after a loss of control ($p = 0.0156$ for CD38⁺, $P = 0.0781$ for HLA-DR, and $P = 0.1563$ for HLA-DR⁺ CD38⁺). No alterations in the expression of CD25 or CD69 were observed over time in either CD8⁺ (Fig. 6A) or CD4⁺ T-cell populations among LoC individuals and did not differ in expression levels observed in long-term controllers (data not shown). In line with previous reports (26), CD8⁺ T cells

also expressed higher levels of PD-1 post-LoC (P = 0.0156, Fig. 6A), especially in central memory cells ($CD8^+$ $CCR7^-CD45RA^+$), while those levels were maintained in persistent controllers (Fig. 6B). Of note, there was a significantly higher level of $PD\text{-}1^+$ $CD8^+$ T cells with a naive phenotype ($CCR7^+$ $CD45RA^+$) in the individuals with LoC than in persistent controllers. Importantly, this difference was already manifest before LoC, potentially identifying an additional risk marker for subsequent LoC.

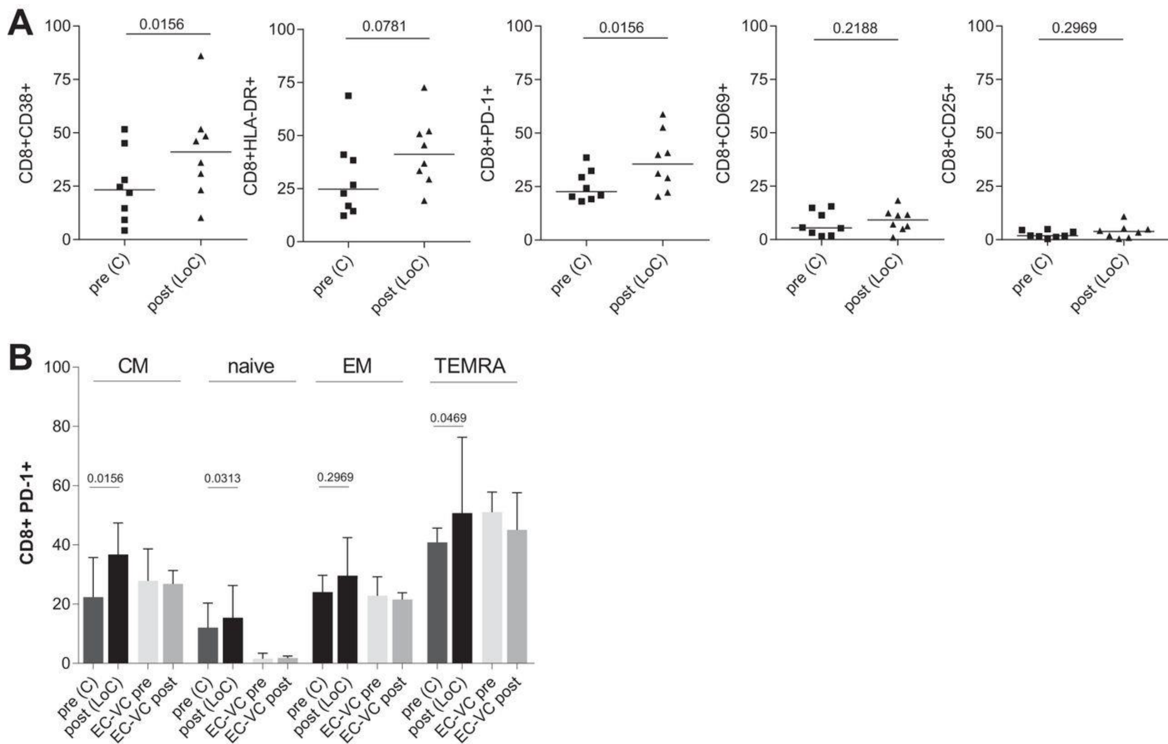


Figure 6. Levels of activation and exhaustion markers. (A) Percentage of expression of CD38, HLA-DR, PD-1, CD25, and CD69 on total CD8+ T cells in individuals that experience a loss of control (n = 8) tested before [pre (C)] or after [post (LoC)] loss of viral control. Values are compared to staining in samples from persistent HIV controllers taken at a median of 102 months apart. Markers were assessed following the gating strategy shown in Fig. S1. P values are shown for Wilcoxon signed rank test. (B) Levels of expression of PD-1 in subsets of $CD8^+$ T cells populations based on CCR7 and CD45RA expression. Median and interquartile range values are shown for a group of 8 individuals that loss of control compared to 8 individuals maintained control over time. The gating strategy used is shown in Fig. S1. P values are shown for Wilcoxon signed rank test.

Discussion

The search for reliable predictors of HIV control has been largely focused on cross-sectional comparisons between individuals with either controlled or uncontrolled HIV infection in the absence of antiretroviral treatment. These studies have yielded a number of correlates of control, many pointing toward an important role of the virus-specific cellular immunity in the natural control of HIV [27–30]. Interpretation of such studies, however, can be limited by its cross-sectional nature and, consequently, by significant

potential differences in host genetics between comparison groups, in particular, HLA class I allele frequencies [8, 31]. In the present study, we have attempted to overcome some of these limitations by analyzing longitudinal samples from subjects who spontaneously lose their previously excellent and longstanding control of viral replication. The identification of markers of control, or loss thereof, could be a helpful tool for the clinical management of individuals off cART with controlled HIV infection and could have important implications for future prevention and treatment strategies in general.

One possible explanation for the abrupt HIV disease progression observed in the present cohort could be HIV superinfection. Superinfection with HIV has been associated with disease progression [17, 32] and has been previously documented, even in the setting of HIV controller cohorts [33–35]. Although superinfection can be facilitated by exposure to a virus that can escape preexisting virus-specific T-cell immunity [33, 36], the first well-documented case of an uncontrolled superinfection in an HIV controller occurred in the presence of a majority of T-cell epitopes being conserved between the first and second infecting virus [17]. Interestingly, in the one case of suspected superinfection in the present cohort, we observed a complete change in the pattern of $CD8^+$ T-cell responses, where only 2 responses were maintained (in Pol and Nef), while 15 new responses toward the whole proteome of HIV were mounted; however, they were evidently not able to control the new virus. Although limited by the cohort size, the present data are in line with earlier reports of highly immunogenic but uncontrolled superinfection and suggest that superinfection may not be a major factor in the sudden loss of virus control in former HIV controllers.

An alternative explanation for LoC could be a switch in the viral tropism from CCR5 to CXCR4, which has been described to precede a decrease in CD4 T-cell counts and progression to AIDS [37–40]. We found that 30% of the individuals (4 out of 13 tested) presented X4-tropic virus after progression, while none was detected during the controlled stages of infection. Interestingly, one of the individuals that showed an X4-tropic virus was the subject that acquired superinfection, suggesting that the second viral infection may have been with an X4-tropic virus. For the remaining 3 subjects who harbored X4 virus post-LoC, it remains unclear whether the change of tropism was either the cause or the consequence of the loss of control. Actually, despite significant advances in the study of relevant parameters involved in HIV control, it is still difficult to define the causality dilemma of certain factors, such as the HIV tropism evolution [28]. Although a switch in coreceptor usage has been attributed to an increase in viral fitness [41, 42], we were unable to obtain any autologous virus from samples drawn at time points when the individuals were controlling the infection and thus could not compare

viral replicative fitness pre- and post-LoC. Cell tropism of HIV has also been associated with cell type-specific differences in antigen processing and, hence, differences in epitope presentation on infected T cells versus infected macrophages or dendritic cells [43]. An R5-to-X4 tropism change could therefore result in an effective escape from T-cell immunity that is mainly focused on epitopes presented by infected T cells but not by macrophages. In addition, it could also explain the marked increase in responses post-LoC in the subject with superinfection and that env responses were only seen after superinfection, although the epitopes were present in wild-type sequence in the controlled virus pre-LoC. As only three individuals showed evidence of a dominant X4-tropic virus post-LoC, we were not able to identify any specific response pattern that could correlate with tropism change during HIV infection progression.

As referred to above, HIV controller cohorts are oftentimes enriched in particular "beneficial" HLA class I alleles, including HLA-B*27, HLA-B*57, and others [6]. Our cohort of LoC is no exception to this, as the frequency of those protective HLA class I alleles (B57/58 and B27) was the same as observed in a locally recruited elite and viremic controller cohort [44]. Furthermore, we also detected broad and strong CTL responses, especially toward Gag, consistent with previous reports in comparable cohorts [2, 45–48]. However, it has also been suggested that HIV controllers with beneficial or nonbeneficial HLA class I alleles may control HIV through different mechanisms, with subjects expressing nonbeneficial alleles, such as HLA-B*35, depending critically on their T-cell responses to durable control [44]. As our cohort only included one subject with a strong nonbeneficial HLA class I alleles, the study of larger cohorts of LoC with unfavorable HLA class I genetics will offer a unique opportunity to determine whether the failures of these responses are indeed critical determinants in LoC. In addition, more in-depth characterization of these responses, including avidity testing and effector function profiling [49, 50], rather than its mere presence/absence pre- and post-LoC, may reveal factors that define the "failure" of these responses post-LoC. We addressed the measurement of functional activity here by conducting *in vitro* inhibition assays (VIA) on samples pre- and post-LoC. Our results indeed showed decreased antiviral *in vitro* capacity of $CD8^+$ T cells before LoC compared to persistently controlling EC. Still, the data suggest that declined VIA activity may precede a loss of control and could serve as a predictor of failing immune control. This is in line with cross-sectional analyses that have associated antiviral capacity of CD8 T-cell in VIA with the rate of HIV disease progression and $CD4^+$ T-cell decline [13, 24]. Thus, monitoring VIA activity in untreated individuals may be a useful tool to initiate cART in a timely manner

and may be especially useful in treatment interruption studies that assess outcomes of therapeutic vaccination and other HIV cure approaches.

Another well-accepted explanation for loss of effective immune control of viral replication *in vivo* is the occurrence of CTL escape mutations in the targeted epitope. Although most tested individuals presented an increased number of HLA-associated escape mutation in their Gag sequence between pre- and post-LoC samples, the differences in HLA footprints over time were not significant. As a single escaped CTL epitope may allow for a loss of control, it would be important to test epitope variants for their true ability to escape CTL recognition. However, we did not have sufficient samples at hand to map all responses and assess the effects of epitope mutations on CTL recognition. In addition, extended deep sequencing covering the entire viral genome and additional sampling time points close to LoC would provide more insights into the role that HLA-associated mutations outside Gag may play in the abrupt the loss of control. Deep sequencing would also reveal low-frequency mutations present that were missed by our analyses and, with additional sampling points, allow the evolution and frequencies of these mutations to be closely followed. As the frequency of individuals experiencing a marked rapid LoC is already low, having closer sampling intervals in larger cohorts will be truly challenging. Due to technical hurdles and sample availability needed to reliably amplify gag sequences in individuals with low or undetectable viremia, we cannot compare the occurrence and frequency of Gag HLA-associated escape mutations seen in our subjects with those in individuals with persistent control. However, existing data show that even in individuals with low levels of viral replication, Gag viral evolution can be detected [45, 51], suggesting that their presence does not necessarily need to lead to LoC.

In line with the loss of antiviral capacity and an increased viral replication *in vivo*, we observed a significant increase in the expression of markers of T-cell activation and exhaustion (in particular, $CD38^{+}$ and PD-1), both of which have been linked to HIV disease progression [9, 46–48, 52–54]. Of note, LoC individuals showed a significantly higher level of PD-1-expressing $CD8^{+}$ T cells even before loss of control compared to individuals that maintained their viral control over time. This was especially marked for $CD8^{+}$ T cells with a naive phenotype and may represent an early sign of LoC during which terminally differentiated T cells may reacquire a naive phenotype [55]. These cells may not be able to cope with ongoing viral replication and immune activation pre-LoC, ultimately leading to full-blown loss of control and elevated viral loads. At the same time, the increased levels of exhaustion markers could also explain the reduced VIA activity seen pre-LoC. Moreover, the enhanced activation and effector phenotype of $CD8^{+}$ T cells

could lead to less proliferative capacity of cells *in vitro* and result in reduced VIA activity seen post-LoC. If validated in further cohorts, our analyses may thus have identified reduced VIA activity and elevated levels of PD-1 expression as predictive markers of loss of control in individuals showing natural control of HIV and may help improve their clinical management. The data may also help guide immune-based therapeutic intervention to achieve a functional HIV cure by defining minimal VIA activities and preferred T-cell phenotypes that may warrant treatment interruptions.

In light of reports showing a predictive value of reservoir size and the rebound kinetics of virus after stopping treatment [56–58] or, in on our case, after LoC, it would have been interesting to determine the size of the viral reservoir in the LoC individuals tested here. However, cell availability did not allow for such analyses. Despite some of these limitations, the present report suggests that individuals who presented an abrupt loss of HIV control had an impaired capacity to inhibit *in vitro* viral replication, maybe due to the increased exhaustion of their CD8+ T cells before LoC. Measuring these parameters may help identify untreated HIV controllers that are at risk of losing control and may offer a useful tool for monitoring individuals during treatment interruption phases included in therapeutic vaccine trials or other cure strategies.

Earlier studies addressing loss of control in previous HIV controllers have also been hampered by the small size of the cohorts of such particular individuals or have attempted to address this question in simian immunodeficiency virus (SIV)-infected macaques [59, 60] while assessing only one or additional aspects of host immunity and their role in persistent virus control [28, 52, 61, 62]. Among these, NK function [54], polyfunctionality [18], and the occurrence of new mutations in relatively conserved viral genes have been proposed to precede the abrupt loss of control. As in our study, these analyses are limited by the difficulty of discerning cause from effect of these events or mechanisms, and further work, possibly in SIV models, may be required to validate the importance of the individual factors identified in predicting LoC.

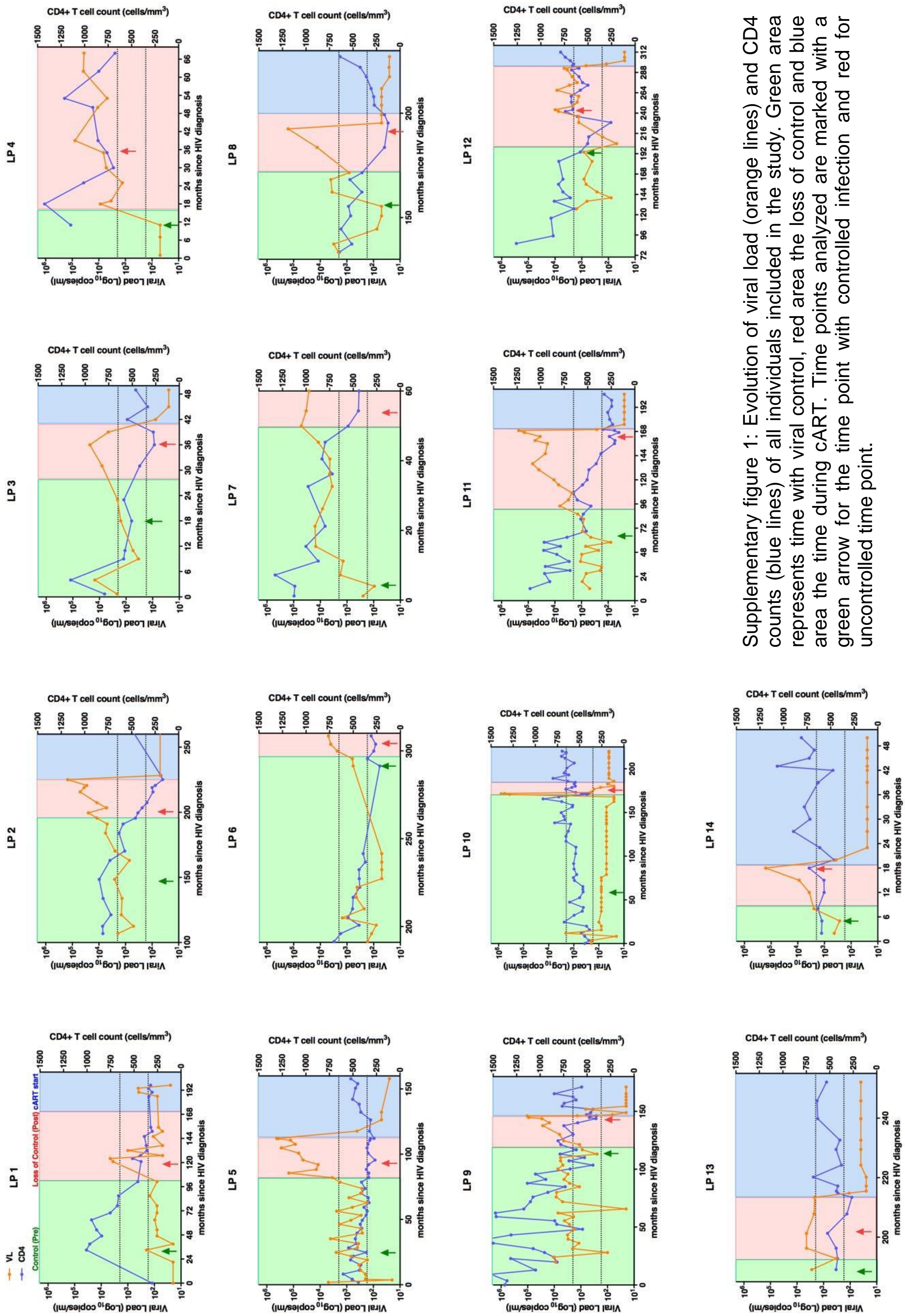
Acknowledgements

We particularly thank the patients in this study for their participation and the HIV BioBank, an integrated part of the Spanish AIDS Research Network, and collaborating centers for the generous provision of clinical samples.

The HIV BioBank is supported by Instituto de Salud Carlos III and Spanish Health Ministry (grant RD06/0006/0035). This work was supported in part by the Spanish FIPSE gant 360737-09, the Barcelona-based HIVACAT program, funding from the European Union’s Horizon 2020 research and innovation program under grant agreement 681137-EAVI2020, and a research agreement with AELIX Therapeutics, Barcelona, Spain.

Christian Brander is the CSO at AELIX Therapeutics.

Supplementary material



Supplementary figure 1: Evolution of viral load (orange lines) and CD4 counts (blue lines) of all individuals included in the study. Green area represents time with viral control, red area the loss of control and blue area the time during cART. Time points analyzed are marked with a green arrow for the time point with controlled infection and red for uncontrolled time point.

```
HXB2       MGARASVLSG GELDRWEKIR LRPGGKKKYK LKHIVWASRE LERFAVNPGL LETSEGCRQI LGQLQPSLQT GSEELRSLYN TVATLYCVHQ
LP1 post   ---------- ----K----- ---------- ---------- ---------- -------K-- -E----A--- -----K---- ---------K
LP2 pre    ---------- -K--Q----- ---------- ---------- ---------- ---------- ---------- -----K---- ----------
LP2 post   ---------- -K--Q----- ---------- ---------- ---------- ---------- ---------- -----K---- ----------
LP3 pre    ---------- -K--K--R-- ---------R ---------- ---------- ---------- ---------- --------F- -I--------
LP3 post   ---------- -K--K--R-- ---------R ---------- ---------- ---------- ---------- --------F- -I--------
LP4 pre    ---------- -Q--K----- -------S-- ---------- ---------- ---------- ------A--- ---------- ---V------
LP4 post   ---------- -K-----R-- -------Q-R ---------- ---------- --------K- IE-------- --------F- ---------E
LP5 pre    ------I--- ---------- ---------- ---L------ --------S- ----D--K-- ---------- -----K-V-- -I------L-
LP5 post   ------I--- ---------Y -------R-- ---L------ ---------- ---AD----- ---------- -----K---- ---------R
LP6 post   ---------- ---------- ---------- ---------- ---------- --------K- ---------- -----K---- ---V------
LP7 post   ---------- ---------- -------Q-R ---------- -----L---- -------K-- ---------- --------F- ---V-----R
LP8 pre    ------I--- ---------- -------Q-R ---L------ ---------- -------K-- IR-------- ---------- ----------
LP8 post   ------I--- ---------- -------Q-R ---L------ ---------- -------K-- IR-------- ---------- ----------
LP9 pre    ---------- ----K----- -------Q-R ---------- ---------- -------KK- -K----A--- ---------- -I--------
LP9 post   ---------- ----K----- -------Q-R ---------- ---------- -------KK- -E----A--- ---------- -I-------R
LP10 post  ---------- EK--S----- ---------R ---L------ -----L--D- ---A---Q-- M-----A--- -T------F- ---V-F---K
LP11 pre   ---------- -K--Q----- ---------R ---------- ---------- ---AG--A-- -T-------- -----K---- ----------
LP11 post  ---------- -K--Q----- ---------R ---------- ---------- ---AK--A-- -T-------- -----K---- -I-V------
LP12 pre   ---------- -K--Q-GE-- ---------- ---------- ---------- I--------- ---------- -----K---- ----------
LP12 post  ---------- -K--Q----- -----R---- ---------- ---------- I--------- ---------- -----K---- ----------
LP13 post  ---------- ---------- --Q------- ---------- ---------- ---A------ ------A--- ---------- ---------K
LP14 pre   ---------- ---------- ---------- ---------- ------D--- ---------- IR-IP----- --------F- ---V------
LP14 post  ---------- ---------- ---------- ---------- ------D--- ---------- IR-IP----- --------F- ---V------
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LP2 pre    ---V------ -E-------- ----------AG--NNS---- ---------- ---------- ---------- ---------- ----------
LP2 post   ---------- -E-------- ----------AG--DNS---- -------L-- ---------- --------I- ---------- ----------
LP3 pre    G--V------ ---V------ ---------- N----S---- -------L-- ---------- --------I- ---------- --T-------
LP3 post   G--V------ ---V------ ---------- N----S---- -------L-- ------L--- --------I- ---------- --T-------
LP4 pre    ---VK----- ---------- ---------- -----S---- -------L-- ---------- ---------- ---------- ----------
LP4 post   K---R---D- ---------- ---------- -A--R----- -------L-- -----P---- --------I- ---------- --T-------
LP5 pre    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
LP5 post   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
LP6 post   K--------- ---------- ---------- ---------- ---------- ------L--- --------I- ---------- ----------
LP7 post   --DI------ ---------- ---S-K---T ---NNS---- ---------- ------L--- --------I- ---------- ----------
LP8 pre    K---R----- ---------- ---------- ---------- ---------- -----PL--- ---------- ---------- ----------
LP8 post   K---R----- ---------- I--------- ---------- ---------- -----PL--- ---------- ---------- ----------
LP9 pre    --D------- -D------- ---G------ ----------- ---------- ---------- ---------- ---------- ----------
LP9 post   --D------- -D------- ---------- ----------- ---------- ---------- ---------- ---------- ----------
LP10 post  -K-------- -E-V-KA-K- GQE-Q-A-MD EGS   --A-- -------A-- ---------- ---------- ---------- ----------
LP11 pre   --DV--Q--- -E-------- ---------- -----S-A-- -------M-- ------L--- --------I- ---------- ----------
LP11 post  --DV--Q--- -A-------- ---------- ---D-S-A-- -------M-- ------L--- --------I- ---------- --T-------
LP12 pre   K--V------ -E-------- ----- ---- -A-NNS---- -------LH- ------L--- --------I- ---------- ---------A
LP12 post  K--V------ -E-------- ----- ---- -T-NNS---- -------L-- -----PL--- --------I- ---------- ---------A
LP13 post  --I------- -E-------- ---------- --R------- --------I- ---------- ---------- ---------- ----------
LP14 pre   KV-------- -E-L----K- -QQMT----- -K-     --- -----K---- ------L--- --------I- ---------- ------D---
LP14 post  KV-------- -E-L----KE -QQTT----- -K-     --- ---------- ------L--- --------I- ---------- ------D---
```

```
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LP1 post   ---------- ---------- ---------- ---------- ---------- ---------- -N-----A-- ---------- ----------
LP2 pre    ---------- ---------- ---------- ---------- --V------- ---------- ---------- ---------- ----------
LP2 post   ---------- ---------- ---------- ---------- --V------- ---------- ---------- ---------- ----------
LP3 pre    ---------- ---------- --D------- ---------- --V------- ---------- ---------- -S-------- ----------
LP3 post   ---------- ---------- --D------- ---------- --V------- ---------- ---------- ---------- ----------
LP4 pre    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
LP4 post   ---------- ---------- ---------- ---------- --V------- ---------- -------A-- -----V---- ----------
LP5 pre    ---------- ---------- ---------- ---------- --V------- ---------- ---------- ---------- ----------
LP5 post   ---------- ---------- ---------- ---------- --V------- ---------- ---------- ---------- ----------
LP6 post   ---------- ---------- ---------- --------Q- --L------- ---------- -N-A--VA-- -G-------- ----------
LP7 post   ---------- ---------- ---------- ---------- ---------- ---------- -N-----A-- ---------- ----------
LP8 pre    ---------- ---------- ---------- D--------- ---------- ---------- ---------- ---------- ----------
LP8 post   ---------- ---------- ---------- D--------- ---------- ---------- ---------- -H-------- ----------
LP9 pre    ---------- ---------- ---------- ---------- --V------- ---------- ---------- ---------- -------M--
LP9 post   ---------- ---------- ---------- ---------- --V------- ---------- ---------- ---------- -------M--
LP10 post  ---------- ---------- --D------- ----M--PQ- ---P---I-- ---------- -N-----T-- ---------- ----------
LP11 pre   ---------- ---------- ---------- ---------- --V------- ---------- -N-------- ---------- ----------
LP11 post  ---------- ---------- ---------- --------Q- --V------- ---------- -N-------- -H-------- ----------
LP12 pre   ---------- ---------- ---------- ---------- --V------- ---------- ---------- ---------- ----------
LP12 post  ---------- ---------- ---------- ---------- --A------- ---------- ---------- ---------- ----------
LP13 post  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
LP14 pre   ---------S ---------- --D---D--- D---T----- ---P------ ---------- ---N---Q-- -S---V---- ----------
LP14 post  ---------S ---------- --D------- ----T----- ---P------ ---------- -N-N---Q-- -S---V---- ----------
```
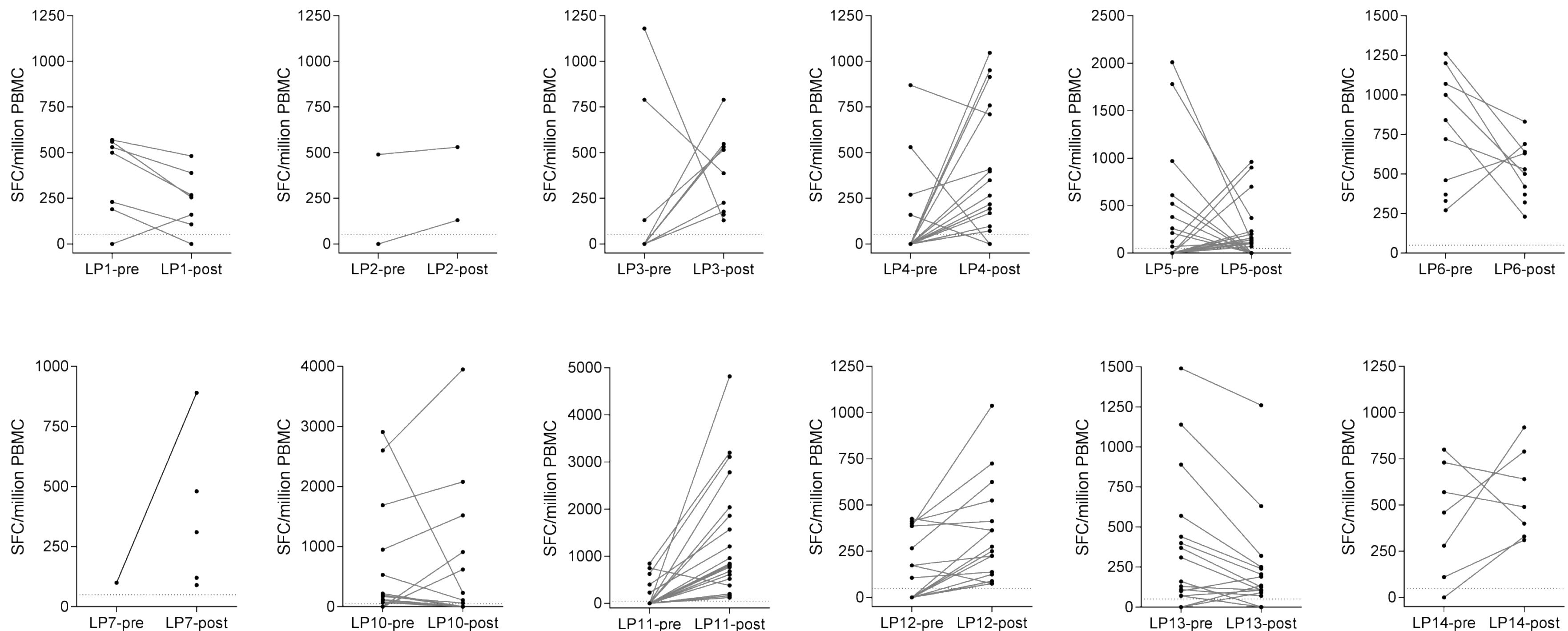

HXB2	NKIVRMYSPT	SILDIRQGPK	EPFRDYVDRF	YKTLRAEQAS	QEVKNWMTET	LLVQNANPDC	KTILKALGPA	ATLEEMMTAC	QGVGGPGH
LP1 post	----------	----------	-S--------	----------	----------	----------	----------	----------	--------
LP2 pre	-------C-V	----------	----------	-**R**--------	----------	----------	----------	----------	--------
LP2 post	-------S-V	-----**K**----	----------	--**V**-------	----------	----------	---------G	----------	------S-
LP3 pre	----------	----------	----------	----------	-D--------	----------	---------G	----------	--------
LP3 post	----------	-----**K**----	----------	----------	-D--------	----------	---------G	----------	------**S**-
LP4 pre	----------	-----K----	----------	-R--------	----------	-----S----	----------	----------	--------
LP4 post	---------V	----------	----------	----------	-**D**--A-----	----------	--------S**G**	-------S--	--------
LP5 pre	---------V	----------	----------	----------	----------	----------	----------	----------	------S-
LP5 post	---------V	----------	----------	----------	----------	----------	-N--------	----------	--------
LP6 post	----------	-----K----	----------	---------**T**	----------	----------	----------	----------	------S-
LP7 post	---------V	----------	----------	----------	----------	----------	----------	----------	--------
LP8 pre	---------V	-----K----	----------	----------	-D--------	----------	----------	----------	--------
LP8 post	----------	-----K----	----------	----------	----------	----------	----------	----------	--------
LP9 pre	----------	-----K----	----------	----------	----------	----------	----------	----------	--------
LP9 post	----------	-----K----	----------	----------	----------	----------	----------	----------	--------
LP10 post	---------V	----------	----------	---------T	----G---D-	----------	----R----G	-S--------	--------
LP11 pre	---------V	----V-----	----------	--I-------	-**D**--------	----------	----------	----------	--------
LP11 post	---------V	----VK----	----------	--I-------	-**D**--------	----------	----------	----------	--------
LP12 pre	---------V	----------	----------	----------	----------	----------	----------	----------	--------
LP12 post	---------V	----------	----------	----------	----------	----------	----------	----------	--------
LP13 post	---------**S**	----------	----------	----------	----------	----------	----------	----------	--------
LP14 pre	---------V	----------	----------	F--------T	----------	----------	----------	----------	--------
LP14 post	---------V	----------	----------	F--------T	----------	----------	----------	----------	--------

HXB2	RVLAEAMSQV	TNSA·TIMMQR	GNFRNQRKIV	KCFNCGKEGH	TARNCRAPRK	KGCWKCGKEG	HQMKDCTERQ	ANFLGKIWPS	YKGRPGNFLQ
LP1 post	----------	-G--A------	----------	----------	--**R**-------	----------	----------	----------	----------
LP2 pre	----------	-S--·N-----	----------	----------	I---------	----------	----------	----------	H-------H-
LP2 post	----------	-S--·NV----	----G---S-	----------	I---------	----------	----------	----------	H-------H-
LP3 pre	----------	--TP·N-----	--Y------	----------	**L**-K-------	----------	----------	----------	NR--------
LP3 post	----------	--TP·N-----	--Y------	----------	**L**-K-------	----------	----------	----------	HR--------
LP4 pre	----------	----·-V----	----------	----------	----------	----------	----------	----------	-R--------
LP4 post	----------	----·-----G	---K--G-T-	-------V--	--K-------	----------	------I---	------V-S-	-R--------
LP5 pre	----------	----·-V----	----------	----------	I-**R**--K----	R---------	----------	----------	----------
LP5 post	----------	----·-V----	----------	----------	I-**R**--K----	R---------	----------	----------	----------
LP6 post	---------A	----·-V----	----S-----	----------	----------	R---------	----------	----------	----------
LP7 post	----------	----·-----K	--S-----SI	------R---	----------	----------	----------	----------	H**R**--------
LP8 pre	----------	----·------	---R------	----------	I-**R**-------	----------	----------	----------	----------
LP8 post	----------	----·------	---R------	----------	I-**R**-------	----------	----------	----------	----------
LP9 pre	----------	-**S**--·------	--------M-	----------	I-**R**-------	----------	----------	----------	----------
LP9 post	----------	----·------	--------M-	----------	I-**R**-------	----------	----------	----------	----------
LP10 post	---------A	- K ·AT---K	S--KGP-RQI	----------	L---------	----------	----------	----------	H---------
LP11 pre	----------	---P·------	----------	----------	I---------	R---------	----------	----------	--------P-
LP11 post	----------	---S·A----K	----------	----------	I---------	R---------	----------	----------	--------P-
LP12 pre	----------	---S·------	----------	----------	I-R-------	----------	----------	----------	--------P-
LP12 post	----------	---S·------	--------P-	----------	I-R-------	----------	----------	----------	--------P-
LP13 post	----------	----·------	----------	------**R**---	----------	----------	----------	----------	H---------
LP14 pre	----------	-STN· -----	S--KS--RM-	-------D--	----------	----------	----------	----------	N---------
LP14 post	----------	-STN· -----	S--KS--RM-	-------V--	----------	----------	----------	----------	N---------

```
HXB2       SRPEPTAPPE ESFRSGVETT TPPQKQEPID KELYP·LTSLR SLFGNDPSSQ
LP1 post   G--------- ----F-E--- --S---GQ-- ---S-·-A--- ----------
LP2 pre    --L------- ----F-E--- --S------- -----·----- ----------
LP2 post   --L------- ----F-E--A ---------- --M--·-A--- ----------
LP3 pre    -----P---- ----F-E--- --S---GQ-- --V--·----- ----------
LP3 post   -----P---- ----F-E--- --S---GQ-- --V--·----- ----------
LP4 pre    ---------- ---GF-G--- --S------- --N--·----- ----------
LP4 post   N--------- ----F-E--- --S----T-- --K--·----- ----------
LP5 pre    ---------- ---------P --S------- -----·----- ----------
LP5 post   ---------- ---------P --S------- -----·----- ----------
LP6 post   --L------- --------M- --S------- -----·----- ----------
LP7 post   ---------- ----F-E--- --S-----K- Q-K--·-A--- ----------
LP8 pre    N--------- ----F-E--- --S------- -----·----- ----------
LP8 post   N--------- ----F-E--- --S------- -----·----- ----------
LP9 pre    ---------- ----F-E--A --S------- -----·----- ----------
LP9 post   ---------- ----F-E--A --S------- -----·----- ----------
LP10 post  ---------A ---GF-E-IA P- L---TKK E----·-A--K ----S-----
LP11 pre   --L------- ----F-E--- --SR-P-L-- -D-S-·-A--- ----------
LP11 post  --L------- ----F-EG-- --SR-P-L-- -D-S-·-A--- ----------
LP12 pre   --L------- ----F-E--- --S------- --M--·----- ----------
LP12 post  --L------- ----F-EG-- --S------- --M--·----- ----------
LP13 post  --L------- ------E--- --S------- ---H-·----- ----------
LP14 pre   N--------A ---GF-E- I ---P---QKT EG---P-A--- R---------
LP14 post  N--------A ---GF-E- I ---P---QKT EG---P-A--- R---------
```

Supplementary figure 2: Alignment of Gag sequences obtained for each individual (pre and post LoC) to HXB2 sequence. HLA-associated footprints are marked in red.



Supplementary Figure 3: Individual HIV-specific responses. Magnitude of CD8+ T cell HIV-specific responses expressed as SFC/ million PBMC is shown longitudinally before and after progression (n=12).

Supplementary table 1: Clinical characteristics of patients with persistent HIV control (long-term controllers).

ID (EC/VC)	Age, years*	Gender	Time since known HIV-1 infection (years)*	Log10 of viral load at 1st Follow-up timepoint studied	Log_{10} of viral load at 2nd Follow-up timepoint studied	CD4 cells/mm^3 at 1st Follow-up timepoint studied	CD4 cells/mm^3 at 2nd Follow-up timepoint studied	HLA A	HLA B	HLA C	HLA DRB	HLA DQB
VC-2515	49	Male	16	UD	2,95	777 (29%)	979 (29%)	0101/3201	4002/5701	0202/0602	0701/1301	0303/0603
VC-9540	63	Female	27	UD	2,83	529 (25%)	503 (29%)	0101/2902	4403/5701	0401/0602	0701/1101	0202/0301
EC-1668	42	Female	15	UD	UD	1201 (40%)	785 (44%)	0201/0205	3905/5701	0602/1203	1301/1601	0502/0609
EC-2143	55	Female	20	UD	UD	717 (33%)	454 (27%)	1101/2402	3501/5201	0401/1202	1301/1502	0601/0603
EC-3901	47	Female	17	UD	UD	1201 (44%)	689 (37%)	0101/0201	0801/5701	0602/0701	0301/0701	0201/0303
EC-1530	50	Male	28	UD	1,68	564 (22%)	706 (31%)	0201/3303	1302/5801	0302/0602	0701/1302	0202/0609
EC-9079	55	Female	24	UD	2,30	645 (43%)	450 (36%)	0301/1101	0702/2705	0102/0702	0301/1101	0201/0301
EC-9080	53	Male	22	UD	UD	440 (25%)	493 (29%)	0205/3101	4901/5101	0701/1502	1201/1301	0301/0603

**Age and HIV duration is shown for the latest timepoint analyzed. EC: elite controller. VC: viremic controller*

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This declaration concerns the following article/manuscript:

Title: Mechanisms of abrupt loss of virus control in a cohort of previous HIV controllers
Authors: Miriam Rosás-Umbert, Anuska Llano, Rocio Bellido, Alex Olvera, Marta Ruiz-Riol, Muntsa Rocafort, Marco A. Fernández, Patricia Cobarsi, Manel Crespo, Lucy Dorrell, Jorge del Romero, José Alcami, Roger Paredes, Christian Brander, Beatriz Mothe.

The article/manuscript is: ■ Published □ Accepted □ Submitted □ In preparation

Full reference of the paper: Rosás-Umbert M, Llano A, Bellido R, Olvera A, Ruiz-Riol M, Rocafort M, Fernández MA, Cobarsi P, Crespo M, Dorrell L, Del Romero J, Alcami J, Paredes R, Brander C, Mothe B. Mechanisms of abrupt loss of virus control in a cohort of previous HIV controllers. Journal of Virology. 2019 Feb 5;93(4). doi: 10.1128/JVI.01436-18.

The phD student has contributed to the elements of this article/manuscript as follows:

A. No or little contribution
B. Has contributed (10-30%)
C. Has contributed considerably (40-60%)
D. Has done most of the work (70-90%)
E. Has essentially done all the work

Formulation/identification of the scientific problem	B
Planning the experiments and methodology design and development	C
Involvement in the experimental work/data collection	C
Interpretations of the results	D
Writing the first draft of the manuscript	E
Finalization of the manuscript and submission	D

The work presented in this Chapter was a project granted by FIPSE applied by Drs Brander and Mothe. The phD student performed relevant background literature search. The phD student contributed to the design of the lab analytical plan of the project and was responsible of managing the samples from different cohorts. As for experimental work, the phD student perfomed INFg ELISpot, viral inhibition assays and flow cytometry experiments. She also contributed in the gag and viral tropism sequencing.

Dr Christian Brander (director)   Dr. Beatriz Mothe Pujadas (director)

CHAPTER II

Virological and immunological outcome of treatment interruption in HIV-1-infected subjects vaccinated with MVA-B

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PLoS One. 2017 Sep 27;12(9)

Abstract

The most relevant endpoint in therapeutic HIV vaccination is the assessment of time to viral rebound or duration of sustained control of low-level viremia upon cART treatment cessation. Structured treatment interruptions (STI) are however not without risk to the patient and reliable predictors of viral rebound/control after therapeutic HIV-1 vaccination are urgently needed to ensure patient safety and guide therapeutic vaccine development. Here, we integrated immunological and virological parameters together with viral rebound dynamics after STI in a phase I therapeutic vaccine trial of a polyvalent MVA-B vaccine candidate to define predictors of viral control. Clinical parameters, proviral DNA, host HLA genetics and measures of humoral and cellular immunity were evaluated. A sieve effect analysis was con- ducted comparing pre-treatment viral sequences to breakthrough viruses after STI. Our results show that a reduced proviral HIV-1 DNA at study entry was independently associated with two virological parameters, delayed HIV-1 RNA rebound ($p = 0.029$) and lower peak viremia after treatment cessation ($p = 0.019$). Reduced peak viremia was also positively correlated with a decreased number of HLA class I allele associated polymorphisms in Gag sequences in the rebounding virus population ($p = 0.012$). Our findings suggest that proviral DNA levels and the number of HLA-associated Gag polymorphisms may have an impact on the clinical outcome of STI. Incorporation of these parameters in future therapeutic vaccine trials may guide refined immunogen design and help conduct safer STI approaches.

Introduction

Effective treatments for human immunodeficiency virus (HIV) infection exist and combination antiretroviral therapy (cART) has resulted in a dramatic decrease in morbidity and mortality. However, cART poses enormous challenges on global implementation and is not free of side effects [1][2][3][4]. Since HIV forms latent viral reservoirs from which the virus reactivates and replicates when treatment is interrupted, cART is a non-curative life-long treatment. Therapeutic vaccination in infected individuals aims to boost adaptive immunity against HIV and help to maintain viral replication at undetectable or low levels in the absence of cART. The safe development of such strategies is complicated by the lack of well-defined parameters of HIV immune control and the uncertainties regarding most suitable endpoints in clinical vaccine trials. While results from cross-sectional cohorts of natural HIV infection point to various immune markers that are associated with viral load, no robust immune parameters have been

identified that could serve as reliable predictors of viral control in patients receiving therapeutic vaccines and interrupting antiretroviral treatment [5][6][7][8].

Past therapeutic vaccine trials have oftentimes included structured treatment interruptions (STI) to assess the efficacy of tested vaccines and used control of viral rebound and/or prevention ofCD4 T-cell decay upon treatment cessation as the primary trial endpoint [9][10][11]. However, STI is not free of risk to the health of infected individuals [12]. Therefore, it is generally only considered in well-controlled clinical trials that exclude patients with low CD4 counts, limit the duration of treatment cessation and use very stringent immune and virological criteria for treatment resumption after vaccine failure. A possibly less harmful approach to STI may be the so-called “monitored anti-retroviral pause” (MAP) where treatment is re- started at a pre-set (low) level of viral replication instead of after a pre-defined period off treatment [13]. In either way though, better predictors of viral rebound during MAP/STI are urgently needed to reduce the risk of conducting unyielding STI and to adjust trial design to maximize vaccination outcome. Here, we tested such potential predictors of vaccine outcome in a recently completed therapeutic vaccine trial, referred to as RISVAC03. This trial was a double-blinded phase I clinical trial that assessed the safety and immunogenicity of an MVA-B candidate vaccine given alone or in combination with disulfiram in chronically infected, cART treated individuals who underwent STI post-vaccination [14][15][16]. In the present work, we sought to integrate host and vaccine-induced virological and immune parameters in order to study possible vaccine exerted effects on rebounding virus, and to define correlates of viral rebound dynamics after STI.

Material and methods

The study was approved by the Ethical Comitee of Hospital Clinic de Barcelona and the trial was registered at Clinicaltrials.gov number: NCT01571466.

Patients and samples

The RISVAC03 study was a phase I double blinded, placebo-controlled therapeutic vaccine trial using an MVA vector expressing HIV-1 antigens from clade B (Bx08 gp120 and IIIB gag/pol/nef) with or without a drug to reactivate latent HIV (disulfiram) in successfully cART-treated, chronically HIV-positive individuals [14]. Of the 30 volunteers that participated in the study, 28 underwent an STI after completing full vaccination regimens consisting of three MVA-B vaccinations given intramuscularly [14]. Cryopreserved peripheral blood mononuclear cells (PBMC), plasma and serum samples were stored for immunological and virological studies. Two patients were excluded from

the present analysis; one because of consent withdrawal before vaccination and a second one who did not interrupt ART after vaccination. The 28 participants consisted of 19 subjects in the vaccine arm and 9 in the placebo arm. Samples from pre-treatment time points were available from 13 individuals (11 in vaccine arm, 2 in placebo) and were used for plasma virus sequencing and sieve effect analyses. All individuals had started antiretroviral treatment 6 months or later after infection. High resolution HLA class I typing was performed by DNA sequence-based typing (SBT) and used for an assessment of escape mutations using described HLA footprint data and optimally defined CTL epitope lists as described [17] [18] [19].

IFNg ELISPOT assay

PBMC were thawed and rested for 5 hours at 37˚C before plating 100,000 live cells per well in IFN-γ ELISPOT 96-well polyvinylidene plates (Millipore). PBMC were stimulated with 42 pools of up to 23 peptides as described [14], discriminating HIV proteins that were, or were not, covered by the immunogen sequence contained in MVA-B. Briefly, IFN-γ ELISPOT responses were assessed using 15mer overlapping peptides combined into 25 pools covering the immunogen sequence (Env gp120 n = 6 pools, Gag n = 6, Pol n = 11 and Nef n = 2, referred to as “IN pools”) as well as the rest of the HIV-1 protein sequences not covered by MVA-B (17 pools: Gag p15 n = 1 pool, Pol int = 3, Vif n = 2, Vpr n = 1, Tat n = 1, Rev n = 1; Vpu n = 1, Nef n = 1 and Env gp41 n = 5, “OUT pools”). Un-stimulated cells served as a negative control and phytohemagglutinin (PHA 1μg/ml) stimulation was used as a positive control. Epitope specific, HLA-class I restricted CD8+ T cell responses were measured by determining the targeted optimal epitope based on the subjects HLA class I type, the 15mer reactivity data from previous Elispot screens and the described list of optimal CTL epitopes at the Los Alamos HIV Immunology database [17].

Antibody detection

Plasma levels of anti-HIV native Env-specific IgG antibodies were determined by flow cytometry using MOLT cells expressing native functional Env glycoprotein (from isolates HIV- 1NL4.3 and HIV-1BaL) or lacking Env expression as previously described [20] [21]. IgG binding Ab were quantified using a PE-F(ab)2 Goat anti-human IgG Fc specific (Jackson Immunore-search) as secondary antibody and the signal expressed as the mean fluorescence intensity in the living MOLT cell gate.

Reservoir and residual viremia determinations

Proviral HIV-1 DNA levels in purified $CD4^+$ T-cell fractions were determined to assess the size of the viral reservoir over time. $CD4^+$ T-cell lysates were used to measure the

housekeeping gene RPP30 and total cell-associated HIV-1 DNA by quantitative droplet digital PCR (ddPCR, BioRad) in samples drawn before and after vaccination as well as at 2 and 12 weeks after treatment interruption, respectively [22]. Residual plasma viremia was quantified using Cobas® Ampliprep/Cobas® TaqMan1HIV-1 Test v2.0 (Roche), after ultracentrifugation of up to 14mL of plasma, in samples from visits at 2 and 12 weeks after treatment discontinuation. For the present study, the detection limit was 0.4–0.8 copies/mL.

Sieve analysis

Ultra-deep sequencing of the HIV-1 gag gene was done using Illumina® NexteraXT protocol and MiSeq platform with 300 bp paired-end sequencing length [23] [24]. Raw sequence data was processed with Trimmomatic [25] to filter out low quality reads and trim adapter sequences. Good quality sequences were aligned against the HXB2R reference (genbank ID: K03455) using the bwa mem algorithm [26]. Mean depth of coverage of the resulting alignments was approximately 15.000. Amino acid variant calling was performed using a codon- level approach with an in-house pipeline. Amino acid variants were flagged if associated with HLA footprints specific for the patient’s HLA class I genotype. QuasiRecomb [27] software was used to obtain nucleotide positional entropy values and to reconstruct full gag haplotype sequences. Mean entropy values were calculated for Gag epitopes coordinates and the 4 most predominant haplotypes were used for analysis by MUSCLE multiple alignment with HXB2R and TN93+G+o nucleotidic distance calculation within the MEGA software [28].

Statistical analysis

Quantitative data for longitudinal determinations were compared by paired Wilcoxon paired test. Comparisons between vaccinees and placebo recipients were analyzed using Mann-Whitney U test. Correlations were performed using the Spearman rank test. P-values <0.05 were considered to be statistically significant. GraphPad Prism version 4.0c (San Diego, CA) was used.

To define host and vaccine-induced virological and immune parameters that predict timeto viral rebound (defined as weeks until there is detectable plasma viral load (>50 copies/mL)) after treatment interruption and the rebounding peak of viral load, we used two adjusted linear regression models. In both cases we included as independent variables: presence/absence of protective HLA class I alleles [29], MVA-B vaccination, magnitude ofHIV-1 specific T cell responses, disulfiram administration, viral adaptation at CTL epitopes, pre-cART plasma VL, $CD4^+$ T-cell counts and levels of proviral HIV-1

DNA. Linear regression models were adjusted using SPSS software Version 15.0 (SPSS Inc., Chicago, IL), and a p-value of less than 0.05 was considered significant.

Results

Delayed viral rebound in MVA-B vaccinees and increased cellular and humoral specific HIV immune responses after treatment interruption

The recently reported immunological and virological outcome MVA-B based therapeutic vaccination in the trial RISVAC03 demonstrated increased Gag-specific T cell responses in vaccinees compared to placebo controls [14]. The data also showed a modest but statistically significant delay in viral rebound in the vaccinees, particularly in vaccinated individuals not receiving disulfiram (p = 0.01) [14]. Here, we show that, in contrast to the modest elevation in magnitude of immune responses seen upon vaccination and reported previously[14] [16], there was a major increase in breadth and total magnitude of HIV-1 specific T cell responses after treatment interruption. Total magnitude ofHIV-1 responses increased from a median of 1,235 to 4,054 SFC/106 PBMC in placebos (p = 0.0078, Wilcoxon paired test) and median of 1,577 SFC/106 PBMC at STI start to 4,767 SFC/106 PBMC at 12 weeks after STI (p = 0.049, Wilcoxon paired test, Fig 1A) in vaccinees. The breadth of responses increased as well in both groups from initially a median of 6.5 pools to 10.5 in the vaccinees (p = 0.1531, Wilcoxon paired test) and, more pronounced, to 12.5 in placebo (p = 0.0078, Wilcoxon paired test, Fig 1B). Of note, placebo recipients increased their T cell responses targeting the vaccine insert as well as the rest of the HIV proteome upon treatment interruption (p = 0.0078, p = 0.0234 respectively, Wilcoxon paired test, Fig 1C). In contrast, in vaccinees, responses to targets not covered by the vaccine insert were boosted (p = 0.0067, Wilcoxon paired test, Fig 1C) while responses to regions contained in the immunogen sequence did not further expand.

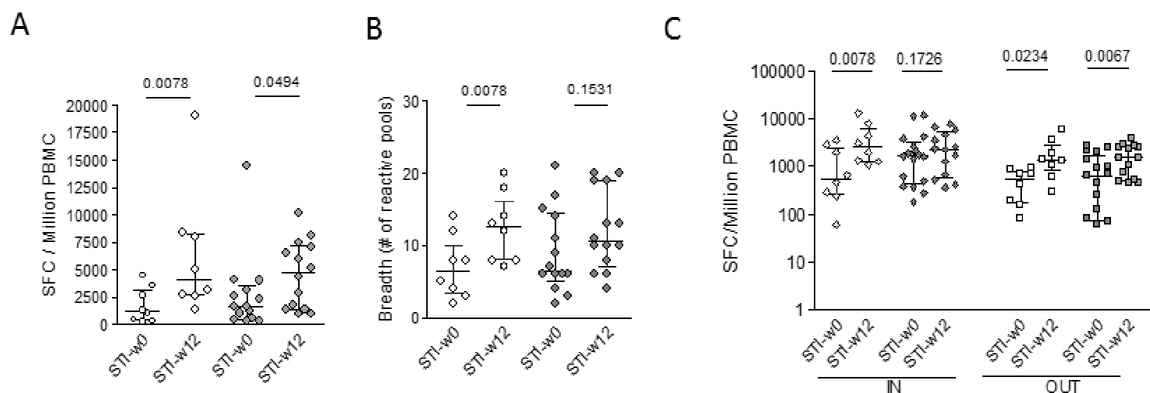

Fig 1. Increased cellular immune responses to HIV after treatment interruption. Magnitude (A) and breadth (B) of T cell responses to the entire HIV-1 proteome at start (STI-w0) and after w12 (STI-w12) of structured treatment in interruption (STI) is shown for placebo recipients (white) and the vaccinated group (grey). Median and interquartile range and p-values (Wilcoxon paired test) are shown. In (C), responses are divided into responses to regions of HIV that are covered (IN) or are not covered (OUT) by the MVA-B vaccine immunogen sequence.

Concomitant with the increase in T-cell responses during treatment interruption, the levels of both, HIVNL43 and HIV_{BaL} Env-specific IgG increased significantly upon 12 weeks of STI compared either to baseline (pre-ART initiation) or STI-start. This was the case regardless whether all subjects were analyzed together (1.2 fold for HIV_{BaL} and 1.44 for HIVNL43, $p< 0.0001$ for both, data not shown) or stratified by placebo (1.2 fold for HIV_{BaL}, $p = 0.0098$; 1.3 times for HIVNL43, $p = 0.0195$) or vaccinees (1.3 fold for HIV_{BaL}, $p = 0.0015$; 1.5 times for HIVNL43, $p = 0.0004$) (Fig 2). Together, these data demonstrate that rebound viral replication is positively associated with increased T- and B-cell responses to HIV, possibly due to antigen driven expansion of these cells.

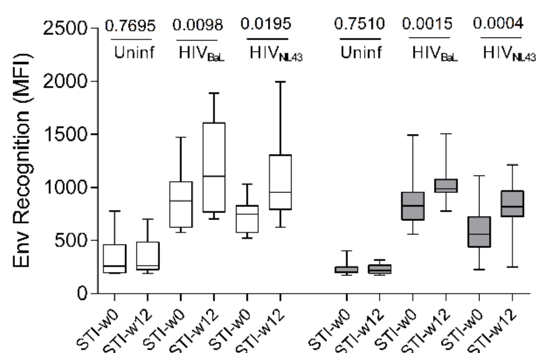

Fig 2. Structured treatment interruption (STI) increases levels of Env specific antibodies. Mean flourescence intensity (MFI) of stained MOLT cells expressing trimeric Env (from isolates HIV-1NL4.3 and HIV-1BaL) or lacking Env expression (uninfected) is show for plasma samples obtained at the start (STI-w0) or after 12 weeks (STI-w12) into STI in the placebo (white, n = 10) or the vaccinated (grey, n = 15) group. P-values for Wilcoxon paired test comparing w0-STI values to w12-STI are shown on top of the figure.

Viral reservoir is replenished after treatment interruption

To determine the effect of therapeutic vaccination on reservoir size and to monitor the extent of a possible replenishment of the viral reservoir upon STI, proviral HIV-1 DNA levels were determined in purified $CD4^+$ T-cell fractions from 18 individuals (13 vaccines and 5 placebo) for whom sufficient PBMC material was available. There was no increase of proviral DNA at week 2 after STI although half of the patients had already experienced a detectable rebound in plasma viral load (median pVL 456 HIV RNA copies/mL, IQR: 192–1,515; assessed in 14/28 individuals that underwent STI, including 6 placebo and 8 vaccinees $p = 0.2114$) (Fig 3A). However, the proviral DNA increased in all of the 18

tested subjects by week 12 after STI with a median 3.1 fold increase (median 416 HIV DNA copies per million CD4⁺ T-cells to 1,257 p = 0.0004) (Fig 3A). Of note, proviral DNA levels in CD4⁺ T-cells after 12 weeks of STI correlated strongly with proviral DNA levels before vaccination (Fig 3B, p = 0.0002, r = 0.79). Furthermore, plasma viral load measured after 4, 8 and 12 weeks into STI showed a positive correlation with levels of proviral DNA at 12 weeks of STI (n = 16 at STI-w4 Spearman r = 0.6416 p = 0.0074, n = 12 at STI-w8 Spearman r = 0.7133 p = 0.0092 and n = 8 at STI-w18 Spearman r = 0.9048, p = 0.0020) (Fig 3C). Proviral DNA levels in CD4⁺ T cells before vaccination were also associated with peak of viral load during viral rebound (n = 18, Spearman r = 0.6615 p = 0.0028) (Fig 3D). Although there was no difference in the increase in proviral DNA levels between placebo recipients and vaccinated individuals, these data indicate that rebounding viremia precedes reservoir reseeding. The data also show that pre-vaccination proviral DNA predicts the extent of reseeding of viral reservoir reseeding in CD4⁺ T cells and peak viremia after treatment interruption.

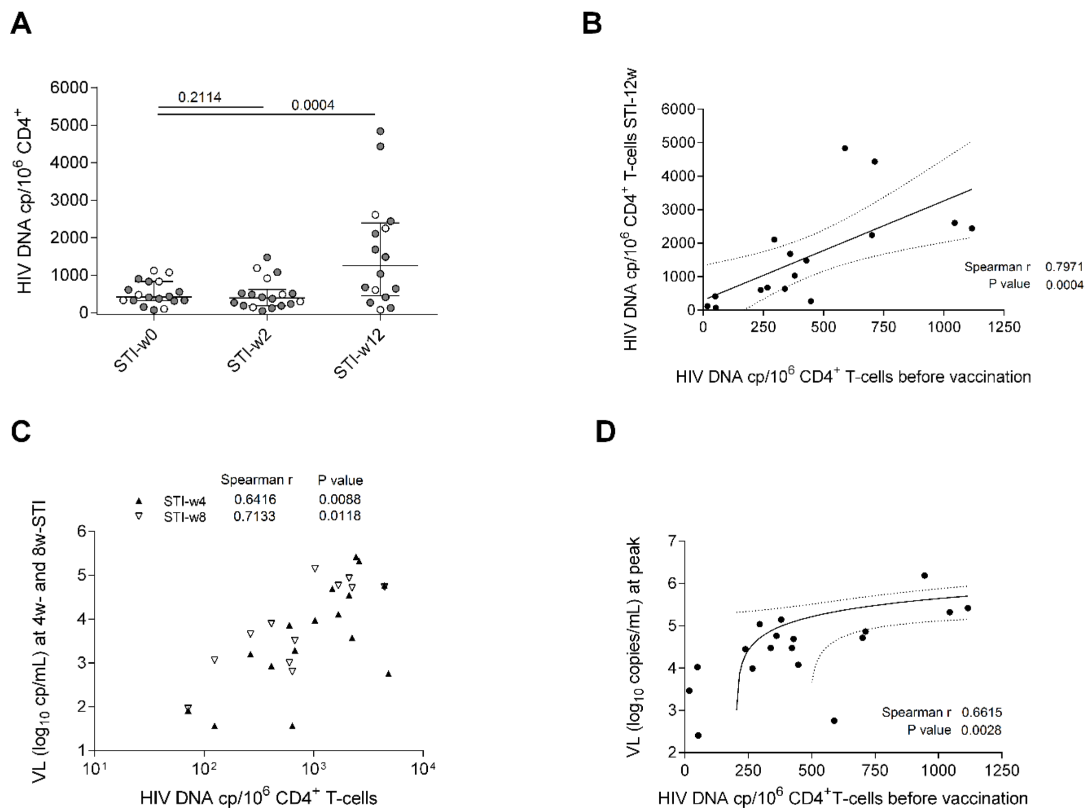

Fig 3. HIV-1 DNA copy numbers in CD4 cells before vaccination predicts extent of viral reservoir replenishment and plasma viral loads after structured treatment interruption (STI). (A) HIV DNA copy numbers in PBMC-derived, purified CD4⁺ T cells at start of STI (STI-w0), and 2 (STI-w2) or 12 (STI-w12) weeks after start of STI in placebo (white) and vaccinated individuals (grey). Median copy number (with interquartile range) is shown in all conditions (p-values Wilcoxon paired test). (B) Correlation between HIV DNA copy number in purified CD4⁺ T cells before any vaccination and after 12 weeks into STI (n = 16). Spearman correlation coefficient and p-value are shown. Linear regression line with 95% confidence intervals is represented. (C) Correlation between HIV DNA copy numbers per 106 CD4 T cells at 12 weeks

into STI and plasma viral loads (log10 copies/mL) 4 or 8 weeks after start of STI. Viral load at 4 weeks into STI (n = 16) is shown in white triangles and at 8 weeks (n = 12) in black triangles (r and p-value are shown for Spearman correlation). (D) Correlation between peak of viral load (log10 copies/mL) during STI and HIV DNA copies detected before vaccination. Spearman correlation coefficient and p-value are shown. Linear regression line with 95% confidence intervals is represented.

No evidence for a strong immune selection pressure on rebounding viral population during STI

To determine whether the vaccine-induced immune response exerted a noticeable selection pressure on rebounding virus, we conducted a sieve effect analysis on the expanding viral populations after STI. Deep sequencing of viral gag RNA was performed in plasma samples obtained pre-cART treatment start (n = 12 individuals (2 placebos, 10 vaccinated subjects with a total of29 (5 + 24) sequences) and at 2 or 12 weeks after STI when viremic plasma samples were available (n = 26 individuals, 8 placebos and 18 vaccinated subjects with a total of 105 (24 + 81) sequences). The phylogenetic distance to a reference sequence (HXB2) and entropy values were determined. The analysis showed no difference of pairwise distances of Gag sequences to the reference sequence HXB2 between placebo and vaccinated group, neither for time points before starting any cART or during STI (Fig 4A). Similarly, there were no differences in the entropy values between placebo recipients and vaccinees and between baseline sequences (before any cART) and after STI (data not shown). Thus, even though vaccination with MVA-B was immunogenic, these responses did not exert a measurable immune selection pressure on the rebounding viruses. To further assess whether the rebounding virus showed signs of successful CTL escape on a single epitope level, the number of HLA associated immune escape mutations were determined based on the individual's HLA class I genotype and compared between samples pre-cART and after STI. There was no increase in the number of HLA class I allele-specific poly- morphisms in the Gag sequences of the rebounding virus when compared to samples drawn pre-cART initiation (Wilcoxon paired test p = 0.3833 for placebo, p = 0.5469 for vaccinated group) and no differences between placebo and vaccines recipients for either time point (Mann-Whitney test p = 0.3685 for samples before cART, p = 0.2058 for samples during STI) (Fig 4B). In fact, 78% of Gag escape polymorphisms (35 out of the 45 detected in 13 subjects available for analysis) were already present in the viral sequences before starting cART while only 6.5% of polymorphisms (n = 3) appeared after STI and 15.5% (n = 7) were lost in between the two time points. In three individuals with available PBMC samples, we were able to relate the occurrence of mutations in T-cell epitopes with a reduced

stimulation of epitope-specific T cells, but these analyses were too limited to draw broader conclusions (data not shown).

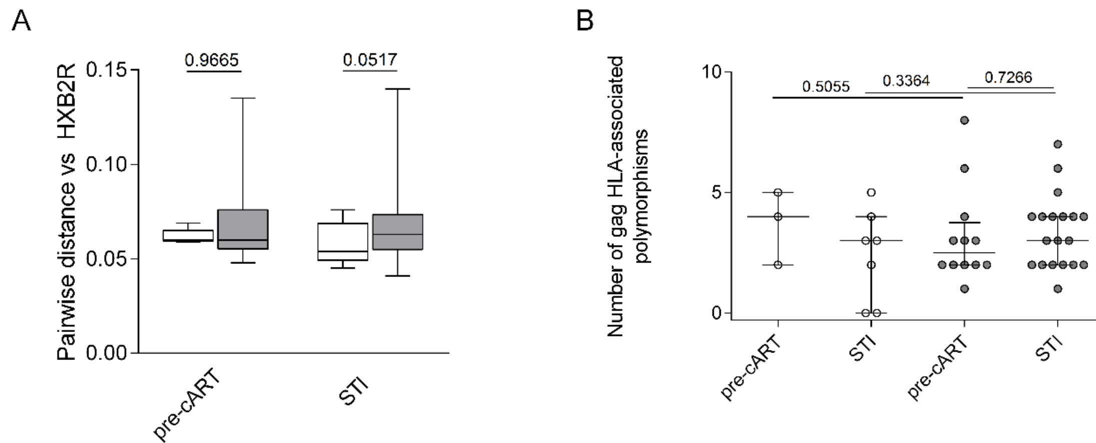

Fig 4. No evidence of immune selection pressure in rebounding virus after therapeutic MVA-B vaccination. (A) Pairwise distances to the reference sequence HXB2 is shown for samples obtained before ART initiation and after 2–12 weeks of treatment interruption. P-value for Mann Whitney test between defined groups (placebo and vaccinated) is shown. (B) The number of HLA-associated polymorphisms in Gag is shown for sequences obtained before cART (n = 3 placebo, n = 12 vaccines) and during viral rebound after STI (n = 7 placebo, n = 19 vaccines). P-value is shown for Wilcoxon paired test when comparing between pre-cART and STI data and p-value for Mann Whitney test is shown to compare groups.

Proviral DNA is associated with time to rebound and peak of viral load after treatment interruption

To determine whether individual or combinations of the above markers had the power to predict viral load kinetics and plasma viral load set point (i.e. pVL at STI-w12), we integrated all data generated from all subjects in a linear regression model that included: presence of protective HLA class I alleles (B*57, B*27 or B*51), receipt of MVA-B vaccination/placebo, total magnitude of HIV-1 specific T-cell responses, disulfiram administration, number of HLA footprints in Gag, pre-cART pVL, CD4 cell counts and levels of proviral HIV-1 DNA before STI. The results show that the only variable that was independently associated with delayed time to rebound (measured as weeks until there is a detectable plasma viral load) was proviral HIV-1 DNA before any vaccination (p = 0.029) (Table 1). In this model, an increase of 100 copies in the number of proviral HIV-1 DNA/106 CD4 T cells decreases the time to rebound by a mean of 2 days. Taking in account the size of our dataset and considering 95% confidence levels, there was no multivariate model emerging that could predict the time of viral rebound. Nevertheless, there was a model that predicted peak of viremia after treatment interruption and which included proviral pre-vaccination HIV-1 DNA (p = 0.019) and the number of HLA-associated polymorphisms in Gag (p = 0.012) (Table 2). In this model, one additional

HLA-associated Gag escape mutation increases the mean peak VL by 114,290 copies/ml, while an increase of 100 copies of HIV DNA/10^6 CD4 augment the mean viral load by 55,900 copies/ml.

Table 1. Regression model for "time to rebound”. Vaccine /placebo arm

Univariate model	Coeff	P-value	95%CI-lower	95%CI-upper
Vaccine /placebo arm	1,228	0,103	-0,265	2,721
Disulfiram/ no disulfiram administration	-0,075	0,919	-1,579	1,429
HLA protective / non-protective	-0,429	0,551	-1,888	1,031
Viral Load pre cART	0,000	0,725	0,000	0,000
CD4 w0-STI	-0,001	0,360	-0,003	0,001
Magnitude w0-STI (SFC/10^6 PMBC)	0,000	0,762	0,000	0,000
Magnitude w12-STI (SFC/10^6 PMBC)	0,000	0,572	0,000	0,000
Proviral DNA before vaccination (HIV DNA copies/10^6 CD4 cells)	-0,003	0,029	-0,005	0,000
Residual Viremia baseline	0,094	0,650	-0,338	0,526
Number of gag HLA-associated escape mutations	-0,926	0,751	-6,887	5,036

Regression model for the dependent variable "time to rebound” (weeks until detectable plasma viremia) was performed on all the individuals undergoing STI (n = 28) and including the following covariates: harboring protective HLA alleles (B*27, B*57 and B*51 (n = 14)), MVA-B vaccination (n = 19), magnitude of HIV-1 specific T cell responses at STI-start (n = 26) and after 12 weeks into STI (n = 24), disulfiram administration (n = 12), CTL virus adaptation (number of total Gag polymorphism and number of HLA-associated escape mutations in Gag) (n = 26), pre-HAART pVL (n = 25), CD4 cell counts (n = 28), residual viremia (n = 18) and levels of proviral HIV-1 DNA at the beginning of the study (n = 18). Coefficients and p-values are shown.

Table 2. Univariate and multivariate regression model for "peak of viral load at rebound”. Vaccine/placebo

	Univariate model				Multivariate model			
	Coeff	P-value	95%CI-lower	95%CI-upper	Coeff	P-value	95%CI-lower	95%CI-upper
Vaccine/placebo arm	-6902.047	0.955	-258614.004	244809.91				
Disulfiram/ no disulfiram administration	166864.439	0.150	-64263.410	397992.29				
HLA protective / non-protective	78599.429	0.494	-154381.726	311580.58				
Viral Load pre cART	-0.016	0.955	-0.594	0.564				
CD4 w0-STI	-107.151	0.555	-475.123	260.822				
Magnitude w0-STI	-0.044	0.998	-35.594	35.506				
Magnitude w12-STI	-4.746	0.783	-40.192	30.700				
Proviral DNA baseline	551.160	0.033	51.021	1051.298	558.952	0.019	28853.106	199727.531
Residual Viremia baseline	6247.651	0.880	-80093.639	92588.941				
Number of gag HLA-associated escape mutations	63348.616	0.080	-8278.874	134976.107	114290.319	0.012	107.529	1010.375
Time to rebound	-45543.989	0.142	-107383.229	16295.250				

Regression model for the dependent variable "peak of viral load at rebound” was performed including the following covariates: harboring protective HLA alleles (B*27, B*57 and B*51 (n = 14)), MVA-B vaccination (n = 19), magnitude of HIV-1 specific T cell responses at STI-start (n = 26) and after 12 weeks into STI (n = 24), disulfiram administration (n = 12), CTL virus adaptation (number of total Gag polymorphism and number of HLA-associated escape mutations in Gag) (n = 26), pre-HAART pVL (n = 25), CD4 cell counts (n = 28), residual viremia (n = 18) and levels of proviral HIV-1 DNA at the beginning of the study (n = 18). Coefficients and p-values are shown for a univariate and multivariate model.

Discussion

Predictors and immune markers for successful outcome of therapeutic HIV vaccination have not been defined, partially due to the lack of strong clinical effects of such therapeutic immune interventions to date. A detailed analysis of the recently completed RISVAC03 trial provided the opportunity to test some virological and immune parameters that could predict the clinically modest, but statistically significant delay in viral rebound seen in vaccinees in this trial. In RISVAC03, all but two individuals who underwent treatment interruption rebounded plasma viral load and had to restart cART [14]. Neither the vaccine-induced increase in Gag-specific T cell responses nor $CD4^+$ T-cell counts before treatment interruption were associated with the time to rebound or levels of peak viral load during STI. We here added markers of virus-specific T- and B-cell immunity, phylogenetic studies of the viral population pre/post STI and detailed reservoir determinations to the analysis of viral rebound kinetics.

CTL and B cell responses increased rapidly in breadth and total magnitude during the treatment interruption in both, vaccine and placebo arms, which is likely explained by the resurgence of viral antigen driving these responses [9][30][31][32]. Since the vaccine-induced responses have been shown previously to be of an effector memory phenotype [16] and to include non-functional $CD8^+$ T cells with elevated PD-1 expansion [15], the T cell responses expanded by rebounding virus may be pre-existing, previously ineffective T cells, unable to control viral replication. A failure to expand functionally active and non-exhausted T cells in the RISVAC03 trial may also explain the failure to observe a significant selection pressure on the rebounding virus [33][34][35]. However, it is also important to note that CTL epitope mutations were largely present in samples from pre cART already and that the source of rebounding virus was already highly adapted viral species. This is in line with viral adaptation to cellular immune responses during primary infection and reflective of a population treated during chronic phase of HIV infection [36][37][38][39][40][41][42]. However, sieve effect analyses in the therapeutic setting may be different from preventive vaccine studies. For instance, a post-hoc analysis of the STEP vaccine trial [33][43][35] showed that breakthrough infections in vaccinated individuals harbored viral sequences that had a significantly greater phylogenetic distance to the vaccine immunogen sequence when compared to viral sequence in individuals that had received placebo. Similar sieve effect analyses have also been conducted, among others, in the RV144 and in the therapeutic DC-TRN vaccine trials [11][44][45] and provide some evidence of vaccine-induced immune selection pressure.

In the present study, half of the patients rebounded plasma viral load rapidly and had detectable plasma viremia after 2 weeks of STI. However, levels of proviral reservoir did not show an increase at that point in either the vaccines or the placebo, suggesting that the viral reservoir, at least in the peripheral blood compartment, has slower kinetics of replenishment than kinetics of viral load rebound detected in plasma. This may offer a window of opportunity to prevent complete reseeding of reservoir, at least in individuals treated in chronic stage, if viral load measurements are conducted with short intervals and cART is resumed immediately after viral rebound is detected. This argues in favor of monitored antiretroviral pause (MAP) that reintroduces ART as soon as viral rebound occurs rather than STI strategy when assessing the efficacy of an HIV therapeutic vaccine [46].

Finally, we analyzed the variables associated with viral rebound at STI week 12 using linear regression models and identified baseline proviral DNA before vaccination as a predictor of the time to viral rebound and peak of viral load after treatment interruption. Similar observations have been made in a study by Li who used a therapeutic rAd5 HIV-1 Gag vaccine [47] [48] and in studies that assessed the outcome of treatment interruptions in people with acute HIV infection [49] [50], showing higher levels of HIV DNA levels to be associated with more rapid viral rebound. However, a meta-analysis of six AIDS Clinical Trials Group STI studies [51] suggests that additional biological factors participate in a complex relationship that define the outcome of STI studies. The participation of additional factors aside from reservoir size is also highlighted by recent clinical examples where in the absence of a targeted immune therapy and even in the absence of undetectable viral reservoirs, viral rebound can occur [52] [53]. Combinational approaches, including ‘kick and kill’ strategies may thus be necessary for a functional cure, where latency reversal agents (LRA), neutralizing antibodies or innate immune stimulators would be administered in combination with therapeutic T cell vaccines to boost the host’s antiviral immunity [54]. This is supported by recent data in SIV-infected rhesus monkeys where a therapeutic vaccination together with toll-like receptor 7 (TLR7) stimulation improved virological control and delayed viral rebound following ART discontinuation [55].

In conclusion, our data provide insights into factors of viral rebound in a therapeutic vaccine trial that showed a statistically significant delay in viral rebound kinetics in vaccinated individuals. Although the data will need to be validated in larger clinical trials, they may further our understanding of potential mechanisms of vaccine control and help improve safety of future clinical trials. Such analyses would further benefit from the inclusion of more immunogenic vaccines as the signals of HIV escape seen here may

be more pronounced and may offer the opportunity to identify novel immune correlates of sustained viral control.

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This declaration concerns the following article/manuscript:

Title: Virological and immunological outcome of treatment interruption in HIV-1-infected subjects vaccinated with MVA-B
Authors: Miriam Rosás-Umbert, Beatriz Mothe, Marc Noguera-Julian, Rocío Bellido, Maria C. Puertas, Jorge Carrillo, Cristina Rodriguez, Núria Perez-Alvarez, Patricia Cobarsí, Carmen E. Gomez, Mariano Esteban, Jose Luis Jimenez, Felipe García, Julià Blanco, Javier Martinez-Picado, Roger Paredes, Christian Brander

The article/manuscript is: ■ Published ☐ Accepted ☐ Submitted ☐ In preparation

Full reference of the paper: Rosás-Umbert M, Mothe B, Noguera-Julian M, Bellido R, Puertas MC, Carrillo J, Rodriguez C, Perez-Alvarez N, Cobarsí P, Gomez CE, Esteban M, Jímenez JL, García F, Blanco J, Martinez-Picado J, Paredes R, Brander C. Virological and immunological outcome of treatment interruption in HIV-1-infected subjects vaccinated with MVA-B. PLoS One. 2017 Sep 27;12(9). doi: 10.1371/journal.pone.0184929.

The phD student has contributed to the elements of this article/manuscript as follows:

A. No or little contribution
B. Has contributed (10-30%)
C. Has contributed considerably (40-60%)
D. Has done most of the work (70-90%)
E. Has essentially done all the work

Formulation/identification of the scientific problem	B
Planning the experiments and methodology design and development	B
Involvement in the experimental work/data collection	B
Interpretations of the results	D
Writing the first draft of the manuscript	E
Finalization of the manuscript and submission	D

The work presented in this Chapter was a project granted by amfAR applied by Drs Brander and Mothe. The phD student performed relevant background literature search. The phD student contributed to the design of the lab analytical plan of the project and was responsible of managing the samples from different clinical sites that participated in the trial. As for experimental work, perfomed INFg ELISpot and contributed to the viral sequencing. The phD student created a database of described HLA polymorphisms and performed analysis of HLA-associated mutations in viral sequences.

Dr Christian Brander (director) Dr. Beatriz Mothe Pujadas (director)

CHAPTER III

HIVconsv vaccines and romidepsin in early-treated HIV-1-infected individuals: Safety, immunogenicity and effect on the viral reservoir (study BCN02)

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(submitted)

Abstract

Kick&kill strategies combining drugs aiming to reactivate the viral reservoir with therapeutic vaccines to induce effective cytotoxic immune responses hold potential to achieve a functional cure for HIV-1 infection. Here, we report on an open-label, single-arm, phase I clinical trial, enrolling 15 early-treated HIV-1-infected individuals, testing the combination of the histone deacetylase inhibitor romidepsin as a latency-reversing agent and the MVA.HIVconsv vaccine. Romidepsin treatment resulted in increased histone acetylation, cell-associated HIV-1 RNA, and T-cell activation, which were associated with a marginally significant reduction of the viral reservoir. Vaccinations boosted robust and broad HIVconsv-specific T cells, which were strongly refocused towards conserved regions of the HIV-1 proteome. During a monitored ART interruption phase using plasma viral load over 2,000 copies/ml as a criterium for ART resumption, 23% of individuals showed sustained suppression of viremia up to 32 weeks without evidence for reseeding the viral reservoir. Results from this pilot study show that the combined kick&kill intervention was safe and suggest a role for this strategy in achieving an immune-driven durable viremic control.

Introduction

Current antiretroviral therapy (ART) effectively suppresses HIV-1 replication, thus preventing disease progression. However, the infection remains chronic given that a latent HIV-1 reservoir, established early after infection, persists despite suppressive ART [1]. Upon ART discontinuation, integrated replication-competent proviruses in the reservoir drive a rapid viral rebound [2]. Therapeutic vaccination has been proposed as a possible approach to induce an effective immune control able to contain rebounding virus.

Most therapeutic vaccines tested expressed one or several HIV-1 proteins, which expanded HIV-1-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses to varying levels. However, the responses were ineffective in controlling viremia after ART interruption, likely because of their suboptimal magnitude, breadth, width, specificity, and/or polyfunctionality [3–7], raising the need for novel immunogens and delivery methods to tackle HIV-1 diversity and the virus' ability to escape. These strategies aim at either increasing the perfect vaccine match of potential T-cell epitopes (PTE) to circulating viruses using multivalent mosaic immunogens designed *in silico* [8, 9], or focusing CTL responses towards more conserved and protective regions of the virus,

which are less likely to mutate and escape the T-cell response [10–14]. Among the latter, the HIVconsv immunogen is one of the most advanced vaccine candidates in clinical development and consists of a chimeric protein assembled from 14 highly conserved domains derived from HIV-1 genes Gag, Pol, Vif, and Env alternating, for each domain, the consensus sequence of the four major HIV-1 clades A, B, C and D [11]. Upon delivery to both HIV-1-negative and positive individuals by heterologous prime/boost regimens as DNA or in simian adenovirus of chimpanzee origin (ChAdV) and poxvirus MVA vectors, HIVconsv vaccines were safe and induced $CD8^+$ T cells with broad inhibitory capacity of HIV-1 *in vitro*, but showed no effect on the viral reservoir [15–22].

To overcome the limitations of therapeutic vaccines in targeting the viral reservoir, vaccines are combined with latency reversing agents (LRA) in the so-called kick&kill strategies [23]. This approach intends to activate transcription of HIV-1 using small molecules able to disrupt the viral latency and facilitate effective sensing and clearance of infected cells by vaccine-elicited HIV-1-specific CTL [24, 25]. Histone deacetylase inhibitors (HDACi) have been proposed as potential HIV-1 LRA [26–28]. Romidepsin (RMD; Istodax®, Celgene Ltd.) is a potent HDACi approved for the treatment of cutaneous and peripheral T-cell lymphomas, which has been shown to induce HIV-1 transcription both *in vitro* and *in vivo* [29, 30]. The REDUC trial combined RMD with Vacc-4x and rhuGM-CSF in chronically suppressed HIV-1-positive individuals, resulting in a significant mean reduction of 39.7% in total HIV-1 DNA [31]. However, this intervention failed to delay viral rebound after ART interruption, suggesting that the reservoir-purge effect was not sufficient and/or the vaccine-induced response was unable to eliminate cells actively replicating HIV-1. In fact, the increase in cell-associated HIV-1 RNA was inversely correlated with time to rebound, supporting that, in the absence of an enhanced HIV-1 specific CTL response, viral reactivation might facilitate viral rebound once ART is interrupted [32].

Here, in this single-arm, open-label, phase I, proof-of-concept study, referred to as BCN02 trial (NCT02616874), we assessed the safety, tolerability, immunogenicity and effect on the viral reservoir of a kick&kill strategy consisting of the combination of HIVconsv vaccines with RMD in suppressed HIV-1-infected early-treated individuals. Participants were rolled-over from the therapeutic vaccine trial BCN01 (NCT01712425), in which individuals who started ART during acute/recent HIV-1 infection had received a prime/boost regimen of the ChAdV63.HIVconsv and MVA.HIVconsv vaccines (CM) [19]. Three years after, BCN01 participants who had shown sustained viral suppression and who accepted to participate in BCN02 study were immunized with two doses of

MVA.HIVconsv, before and after three weekly-doses of RMD, followed by a monitored antiretroviral pause (MAP) for a period of 32 weeks to assess the ability of the intervention to control viral rebound.

Material and Methods

Study design and interventions

The BCN02 clinical trial was a phase I, open-label, single-arm, multicenter, single-country study to assess the safety, tolerability and efficacy of a combined kick&kill strategy in suppressed HIV-1-infected patients that had initiated ART during acute/recent HIV-infection. Individuals were rolled over from vaccine trial BCN01 [19] and invited to participate after 3 years on suppressive ART. Complete list of inclusion/exclusion criteria is available in the Study Protocol (Appendix). The study took place between February 2016 and October 2017 at two HIV-1 units from university hospitals (Hospital Universitari Germans Trias i Pujol -HUGTIP, Badalona and Hospital Clínic, Barcelona) and a community center (BCN-Checkpoint, Barcelona). Before inclusion in the study, all participants signed an informed consent previously discussed, reviewed and approved by the Community Advisory Board of the Barcelona-based vaccine program (HIVACAT). The study was approved by the institutional ethical review board of the participating institutions (Reference Nr AC-15-108-R) and by the Spanish Regulatory Authorities; and was conducted in accordance to the principles of the Helsinki Declaration and local personal data protection law (LOPD 15/1999). The MVA.HIVconsv vaccine was GMP manufactured at IDT Biologika GmbH, Germany, and supplied for the study under a clinical trial agreement with University of Oxford. Risk of Genetically Modified Organism release to the environment was evaluated by the Spanish Ministry of Environment (B/ES/12/09). RMD was supplied for the study by Celgene Ltd. (Couvet, Switzerland) under an investigator initiated clinical trial contract agreement.

The BCN02 trial design is summarized in Fig. 1A. After their inclusion in the study (week 0), all participants received a first dose of 2x108 plaque-forming units (pfu) of MVA.HIVconsv (MVA_1) administered intramuscularly, followed by three weekly doses of RMD of 5 mg/m2 BSA infused intravenously over 4 hours (RMD_{1-2-3}), and a second dose of 2x108 pfu of MVA.HIVconsv (MVA_2) 4 weeks after RMD_3 to compensate for any potential impairment in the previous vaccine-induced response caused by RMD. Following RMD prescription information, participants received prophylactic antiemetic treatment with ondasetron before and during 3 days after each RMD dose.

Eight weeks after MVA2 (week 17), eligible participants initiated a MAP for a maximum of 32 weeks (MAP_{0-32}) or until any ART resumption criteria were met. To be eligible for the MAP, participants had to maintain undetectable HIV-1 pVL and meet the immune futility criteria, defined as showing a net increase in HIVconsv-specific immune response with MVA_2 boost measured in an *ex vivo* IFN-γ ELISpot assay. During MAP, participants were allowed to choose either HUGTIP or BCN-Checkpoint community center for the follow up visits. Symptoms suggestive of acute retroviral syndrome and sexually transmitted diseases were solicited and viral load was tested using the finger-tip Xpert HIV-1 Qual kit (Cepheid, Sunnyvale, CA, US) in all visits. When a positive result was obtained in the Xpert HIV-1 Qual, participants were called in for a confirmatory quantitative pVL within the next 24 hours. If pVL was confirmed to be over 20 copies/ml, a visit was scheduled three days after to closely monitor viral rebound and be able to offer prompt ART resumption if required. Details on viral load management during MAP are described in a Study Protocol (Appendix). After ART ressumption, participants were followed at 4, 12 and 24 weeks to assure that they re-attained viral suppression.

Study population

BCN02 participants were adult (≥18 years) HIV-1-infected individuals, who had initiated ART less than 6 months after estimated date of HIV-1 acquisition and who had received a prime/boost heterologous vaccination regimen using ChAdV63.HIVconsv-MVA.HIVconsv in the parental BCN01 study [19]. To be eligible for BCN02, participants had to maintain optimal HIV-1 suppression during at least 3 years and $CD4^+$ cell counts ≥500 cells/mm3at BCN02 baseline visit. Main exclusion criteria included active hepatitis B or C, history of AIDS-defining disease, treatment for cancer or lymphoproliferative disease within 1 year before study entry or use of immunosuppressants within the 3 months prior to the screening visit. Concomitant treatment with strong CYP3A4 inhibitors was not permitted but switching ART to a non-boosted integrase-inhibitor raltegravir- or dolutegravir-based regimen at least 4 weeks before baseline visit was allowed for those patients receiving ART with ritonavir or cobicistat at screening.

Study Endpoints

The primary endpoint of this study was to assess the safety, tolerability and the effect on the viral reservoir size of the combined treatment with HIVconsv vaccines and RMD given as a latency reversing agent. Secondary endpoints included the extent and specificity of the CTL response and the effect of the intervention in controlling viral rebound after ART interruption. Other secondary endpoints included RMD pharmacokinetics and the effects of RMD on histone acetylation in lymphocytes,

induction of viral transcription, changes in T-cell activation surface markers, and quantification of plasma viremia.

Safety and tolerability were evaluated by the development of grade ≥3 and serious adverse events. Local and systemic adverse events (AE) were solicited prospectively for a minimum of 7 days following each immunization and RMD administration. Both local and systemic AE were graded according to the Division of AIDS (DAIDS) Table for Grading the Severity of Adult and Pediatric Adverse Events, version 2.0, November 2014, accessible online at https://rsc.niaid.nih.gov/sites/default/files/daids-ae-grading-table-v2-nov2014.pdf. AE were specified as unrelated, unlikely, probably or definitely related to the investigational products by the investigator.

Determination of RMD pharmacokinetics

The concentration of RMD in plasma was determined, for RMD_1, at the end of the infusion (4 hours) and 4.5, 5, 6, 8, 12 and 24 hours post-infusion and, for RMD_2 and RMD_3, at the end of the infusion and at 12 hours post-infusion. RMD concentrations were measured by liquid chromatography-mass spectrometry (LC-MS)/mass spectrometry (MS), according to a validated method. A population pharmacokinetic model for RMD was developed using non-linear mixed-effects modelling with the computer program NONMEM version 7.3 (Icon Development Solution, Ellicot City, MD) [34]. Bayesian estimates of the individual parameters of RMD were used to simulate individual time-concentrations curves for each individual.

Flow cytometry determination of acH3 and activation of T cells

The levels of histone H3 acetylation in lymphocytes were determined by flow cytometry from samples taken before (0 hours) and at the end of each RMD infusion (RMD_{1-2-3}) (4 hours), at 8 and 24 hours (+1 day) RMD_1, and at 72 hours (+3 days) and 7 days after (RMD_{1-2-3}). Cryopreserverd PBMC were thawed 4 hours before use, and 500,000 cells were blocked with 600 ul of PBS/10% FBS for 20 minutes and stained with polyclonal rabbit anti-acetyl histoneH3 (10 µg/ml, MerckMillipore #06–599) or normal rabbit serum (control stain, LifeTechnologies #10510) for 30 minutes. Cells were washed and subsequently incubated with donkey anti-rabbit IgG(H+L) (6µg/mL, LifeTechnologies #A21206) for 30 minutes at room temperature in the dark. Cells were washed, re-suspended in 150 µl PBS and analyzed. ~50,000 events were acquired per sample. The median fluorescence intensity (MFI) for each sample was calculated by substracting the background MFI (isotype control stain) from the anti-acetyl histoneH3 stain.

Activation of T cells was determined based upon HLA-DR expression on $CD3^+$ T cells. Cryopreserved PBMC were thawed, and 1,000,000 cells were stained with CD3 APC-Cy7, CD4 FITC, CD8 BV510 and HLA-DR PE-Cy7 (BioLegend #344818, 300538, 301048 and 307616, respectively). Cells were collected on an LSRII instrument (BD), and data analyzed according to the gating criteria shown in Fig. S2 using FlowJo 10 software.

Quantification of cell-associated (CA) HIV-1 RNA in CD4+ T cells

Cell-associated HIV-1 RNA was quantified in peripheral $CD4^+$ T cells by ddPCR (One-Step RT-ddPCR Advanced Kit for Probes, BioRad) from samples taken before (0 hours) and at the end of each RMD infusion (4 hours), at 8 and 24 hours (+1 day) after RMD_1, and 72 hours (+3 days) and 7 days after $RMD_{1\text{-}2\text{-}3}$. CA HIV-1 RNA was quantified using two different primers/probe sets annealing to the 5'LTR and GAG conserved regions of HIV-1, to circumvent potential primer mismatch in individuals' viral sequence as previously described [50]. HIV-1 transcription levels were normalized to the housekeeping gene TATA- binding protein (TBP) and shown as relative to levels before RMD_1.

Ultra-sensitive determination of residual viremia

To evaluate HIV-1 RNA below 20 copies/ml, 4-8 mL of plasma samples taken before (0 hours) and at the end of each RMD infusion (4 hours), at 8 and 24 hours (+1 day) after RMD1, and 72 hours (+3 days) and 7 days after $RMD_{1\text{-}2\text{-}3}$ were ultracentrifugated at 170,000 g at 4 ºC for 30 minutes and viral RNA was extracted automatically using the m2000sp Abbot device. HIV-1 RNA copies were quantified using the Abbott Real-Time HIV-1 assay (Abbott Molecular Inc.) and in-house calibration curve sets as described [51]. The limit of detection (2 HIV-1 RNA copies/mL) was calculated relative to the plasma volume.

Vaccine immunogenicity

Total HIV-1 and HIVconsv-specific T cells were assessed using *ex vivo* cryopreserved PBMC obtained the day of vaccination and 1 week afterwards, 3 weeks after MVA_1, and 4 weeks after MVA_2 using an IFN-γ-detecting enzyme-linked immunoabsorbent spot assay (ELISPOT IFN-γ Mabtech kit). 15-mer peptides overlapping by 11 amino acid were combined into 6 pools of 32-33 peptides per pool corresponding to the HIVconsv vaccine insert (P1-P6, total n=166 peptides, IN pools) (Fig. 3A) and 12 pools of 39-67 peptides per pool spanning the rest of the HIV-1 viral protein sequences (OUT pools for outside the immunogen, obtained through the NIH AIDS Reagent Program). All peptides pools

were tested in duplicates. The final concentration of individual peptides in the ELISPOT assay was 1.57 µg/ml. Medium only was used as no-peptide negative control in quadruplicate wells, and PHA (50 µg/ml) and a CEF peptide pool (2 µg/ml) consisting of 23 previously defined human $CD8^+$ T-cell epitopes from cytomegalovirus, Epstein-Barr virus and influenza virus (C.T.L. OH, USA) were added as positive controls.

To address the breadth of the vaccine-induced response at the peak immunogenicity time point, an IFN-γ ELISPOT assay with *in vitro* expanded T cells was performed on stored samples from week 10 and 13 to test individual overlapping peptides covering the HIVconsv immunogen sequence (n=166 OLP). Briefly, cryopreserved PBMC were thawed and incubated for 3 hours at 37ºC in R10 before stimulation with an anti-CD3 monoclonal antibody during 2–4 weeks in R10 supplemented with 50 U/ml of recombinant IL-2 [52]. Before their use in ELISPOT assays, the expanded cells were washed twice with R10 and incubated overnight at 37ºC in the absence of IL-2. 100,000 cells were used per well and individual OLP were added at 5 µg/ml as in the direct *ex vivo* assay.

Spots were counted using an automated Cellular Technology Limited (C.T.L., OH, USA) ELISPOT Reader Unit. The threshold for positive responses was set at ≥ 50 SFC/106 PBMC (5 spots per well), > the mean number of SFC in negative control wells plus 3 SD of the negative control wells, or 3x the mean of negative control wells, whichever was higher. To avoid overestimating the breadth of responses, positive responses to two consecutive 15-mer overlapping peptide were counted as one response. The highest magnitude of the sequential responses was taken as the magnitude for each identified response.

Quantification of HIV-1 reservoir

To quantify the size of the peripheral blood proviral reservoir, lysed extracts from CD4+ T cells were used to measure total CA HIV-1 DNA by ddPCR. Primers and probes for the RPP30 cellular gene were used for input normalization.

Statistical analysis

Qualitative variables were represented as mean absolute and relative frequencies, whereas quantitative variables were represented as mean or median and range. Safety endpoints are summarized by the number and percentage of participants reporting local and systemic AE and their grading. The Wilcoxon signed rank test was used to test whether the viral reservoir and the immune parameter changed as an effect of the intervention. The maximum breadth of the T-cell response per individual was estimated

as the number of P1-P6 pools eliciting a positive response throughout the study and the number of individual OLP eliciting a response at peak immunogenicity time point from the mapping assay. Reservoir size and immunogenicity were analyzed using GraphPad Prism (v5.01) for Mac OS X (San Diego, CA).

To evaluate the effect of the intervention on viral rebound, a positive pre-defined efficacy signal was established if at least over 20% of patients remained with pVL below 2,000 copies/ml at week 12 of MAP, considering previous data suggesting that early treatment initiation could favor delayed viral rebound/spontaneous viral control in up to 15% of individuals [53]. However, BCN02 was an exploratory pilot trial and, due to the absence of a control arm and its small sample size, the nature of this study only allowed to detect trends in virological effects, which collectively, could be useful to design future studies. To detect possible factors associated to the viremic control observed during the MAP phase, univariate log-binomial regression models were used [54]. This model uses the logarithm as a link function and is a generalized lineal model for a binary outcome where the error terms follows a binomial distribution. The effect size measure of the model is the relative risk. Because of the low number of MAP-C (n=3), multivariate log-binomial regression models were not fitted. The significance threshold for all univariate analyses was set at a two-sided $\alpha = 0.05$. The analyses were performed with R (55) (v3.0.2).

Results

Participants enrolled in the study

Between February 29th and September 15t^{h} 2016, 15 out of the 22 eligible BCN01 participants were enrolled. Seven declined to participate due to their inability to attend all the scheduled visits. Baseline characteristics of trial participants are summarized in Table 1. All 15 participants received two doses MVA.HIVconsv (MVA_{1-2}) and three doses of romidepsin (RMD_{1-2-3}) as shown in the chronogram (Fig. 1A), and were included in the safety, immunogenicity and reservoir analyses. One participant was not eligible for MAP due to immune futility pre-defined criteria and 14 participants underwent a MAP for a maximum of 32 weeks. Retrospective analyses of stored plasma samples obtained during MAP revealed the presence of antiretroviral drugs in some of the samples of one participant, whose MAP data were censored for the viral rebound kinetics (Fig. 1B).

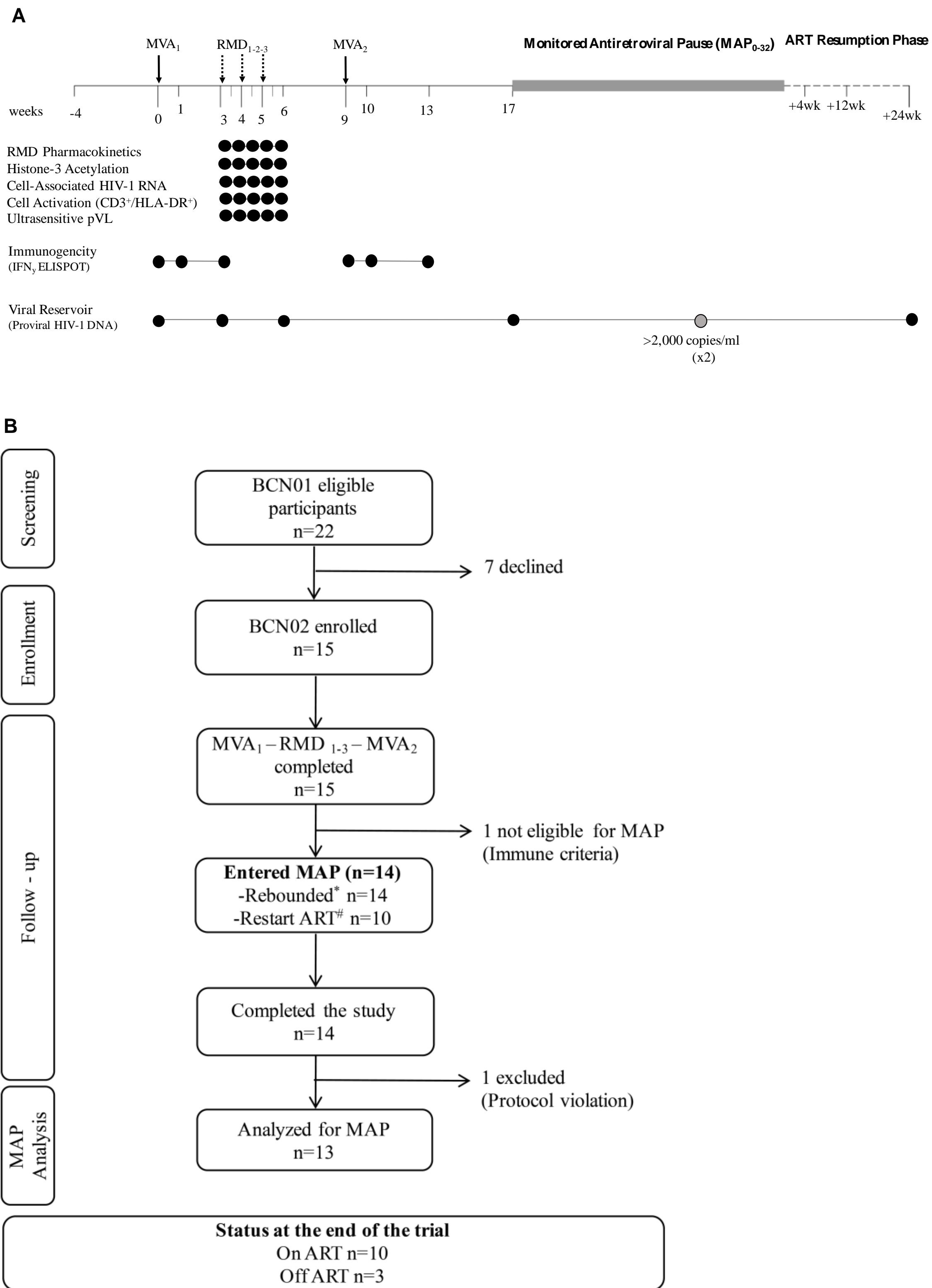

Fig. 1. **Trial design.** (**A**) Schematic study design. (**B**) Consolidated Standards of Reporting Trials (CONSORT) flow diagram for the trial. *Viral rebound during MAP was defined as pVL >20 copies/ml and #criteria for ART resumption included pVL over 2,000 copies/ml in two consecutive determinations, CD4 cell counts decrease over 50% and/or below 500 cells/mm^3 and/or development of clinical symptoms suggestive of an acute retroviral syndrome. MVA, MVA.HIVconsv vaccine. RMD, romidepsin. MAP, monitored antiretroviral pause. ART, antiretroviral therapy. pVL, plasma HIV-1 viral load.

Table 1. Demographic, clinical, and treatment characteristics of study patients at study entry (n=15)	
Age (years)	43 (33 – 51)
Gender (M/F), *n*	14/1
MSM/HTS, *n*	14/1
Time since HIV-1 acquisition to ART (days)	92 (28 – 164)
Pre ART log_{10} HIV-1 RNA (copies/ml)	4.9 (3.2 – 5.8)
Time on ART (years)	3.23 (3.03 – 3.77)
ART regimen, *n (%)*	
TDF/FTC/RAL	11 (73)
ABC/3TC/RAL	2 (13)
ABC/3TC/DTG	2 (13)
$CD4^+$ T-cell counts (cells/mm^3)	728 (416 – 1408)
Ratio CD4/CD8	1.37 (0.97 – 1.93)

Median (range) is shown unless otherwise described. M: male. F: female. MSM: men who have sex with men. HTS : heterosexual. ART: antiretroviral therapy. TDF: Tenofovir Disoproxil Fumarate; FTC: Emtricitabine; RAL: Raltegravir; ABC: Abacavir; 3TC: Lamivudine; DTG: Dolutegravir

Safety of MVA.HIVconsv and RMD administrations

All participants reported adverse events (AE) related to both study investigational medicinal products. A total of 333 AE were recorded during the study intervention phase, 129 after MVA_{1-2} and 204 after RMD_{1-2-3}, which were mostly mild or moderate (grade 1-2) (n=318, 95%). The most frequent AE related to MVA.HIVconsv, summarized in Table 2, were local pain at the injection site and a flu-like syndrome consisting of fatigue, headache, myalgia and/or low-grade temperature (<38°C). Regarding AE related to RMD (Table 3), the most common grade 1-2 events were headache, fatigue, and gastro-intestinal symptoms. Despite prophylactic ondansetron treatment, 4 (27%) patients vomited the days of RMD administration. One participant experienced a grade 4 AE within 4 hours after RMD_3 consisting in a sepsis by *Shigella sonnei*, which was considered as possibly related to RMD, and required hospital admission, thus fulfilling the criteria of serious adverse event (SAE).

No laboratory abnormalities related to MVA_{1-2} were reported. All laboratory abnormalities related to RMD were grade 1-2 (n=22), the most frequent being hypophosphatemia (8

events) and thrombocytopenia (5 events) (data not shown), except from one case of grade 4 creatinine kinase elevation with normal eGFR which resolved. Noteworthy, $CD4^+$ T-cell counts showed a transient decrease by a median of 248 cells/mm^3 three days after each RMD administration which was not fully recovered by day 7 after RMD_3 (Fig. S1). Overall, both MVA.HIVconsv and RMD at the regimen and dose administered in this study were well tolerated and safety profiles were consistent with data previously reported [19, 31].

During the MAP, 12 (86%) participants reported a total of 58 AE, which were all grade 1-2 and not suggestive of acute retroviral syndrome (not shown). Grade 1 anxiety was observed in one participant who repeatedly declined psychological support (participant with protocol violation during the MAP).

Table 2. Summary of adverse events related to MVA.HIVconsv vaccinations (n=15)

	Grade 1, *n*	**Grade 2, *n***	**Grade 3, *n***	**Grade 4, *n***	**Total, *n(%)***
Injection site reaction					
Local pain	7	4	2	0	**13 (87)**
Redness	1	0	0	0	**1 (7)**
Induration	0	0	0	0	**0 (0)**
Systemic adverse events					
Fatigue	7	4	2	0	**13 (87)**
Headache	5	3	1	0	**9 (60)**
Myalgia	4	3	2	0	**9 (60)**
Fever	5	0	0	0	**5 (33)**
Anorexia	3	0	1	0	**4 (27)**
Sweating	2	2	0	0	**4 (27)**
Nausea	2	0	1	0	**3 (20)**
Abdominal pain	0	1	1	0	**2 (13)**
Flatulence	1	0	0	0	**1 (7)**
Somnolence	1	0	0	0	**1 (7)**

Table 3. Summary of adverse events related to RMD_{1-2-3} treatment (n=15)

	Grade 1, *n*	**Grade 2, *n***	**Grade 3, *n***	**Grade 4, *n***	**Total, *n(%)***
Headache	9	5	0	0	**14 (93)**
Fatigue	9	5	0	0	**14 (93)**
Nausea	4	7	0	0	**11 (73)**
Anorexia	8	1	0	0	**9 (60)**
Abdominal pain	5	2	0	0	**7 (47)**
Metallic taste	5	1	0	0	**6 (40)**
Constipation	4	2	0	0	**6 (40)**
Abdominal distension	4	1	0	0	**5 (33)**
Vomits	4	0	0	0	**4 (27)**
Sweating	2	2	0	0	**4 (27)**
Palpitations	3	0	0	0	**3 (20)**
Myalgia	1	1	0	0	**2 (13)**
Rash	0	2	0	0	**2 (13)**
Dry mouth	1	0	0	0	**1 (7)**
ECG: ST-elevation	1	0	0	0	**1 (7)**
Anxiety	0	1	0	0	**1 (7)**
Libido decrease	0	1	0	0	**1 (7)**
Somnolence	1	0	0	0	**1 (7)**
Sepsis by *Shigella sonnei* (SAE)	0	0	0	1	**1 (7)**
Hypotension	0	1	0	0	**1 (7)**

RMD: romidepsin; ECG: electrocardiogram; SAE: serious adverse event

RMD pharmacokinetics and pharmacodynamics

Pharmacokinetics of RMD was comparable to previously described [33]. Each infusion was followed by a rapid and polyexponential decline in RMD concentrations in plasma, reaching nearly undetectable levels by 24 hours after dosing (Fig. 2A). A population pharmacokinetic model adequately describing RMD concentrations in plasma was developed (34). Regarding the direct effect of RMD on chromatin and induction of viral transcription, we found that histone H3 acetylation (acH3) increased rapidly during each RMD infusion, remained high during 4 hours after each dose (Fig. 2B), and returned to baseline values 3 days after RMD_1, which is consistent with previous reports [30, 31]. HIV-1 transcription transiently increased in parallel, with changes more pronounced after RMD_2 and RMD_3 (Fig. 2C). Increases in T-cell activation, measured by proportion of

$CD3^+HLA\text{-}DR^+$ cells, were delayed 3 days after each RMD dose. Over the course of the three RMD doses, T-cell activation increased in a cumulative manner and was maintained up to one week after the last RMD dose (Fig. 2D), suggesting a cumulative effect of RMD.

To evaluate changes in levels of quantifiable plasma HIV-1 RNA, an ultrasensitive single copy assay was used. Kinetics of plasma HIV-1 RNA levels did not follow a clear pattern (Fig. 2E), despite the increasing percentage of participants with detectable low-level viremia at the end of each RMD dose (Fig. S3). Collectively, we reproduced effects on acH3, HIV-1 transcription and T cell activation previously reported in chronically infected individuals [31], suggesting that a lower viral reservoir achieved by early-treatment does not preclude the reactivation potential of RMD.

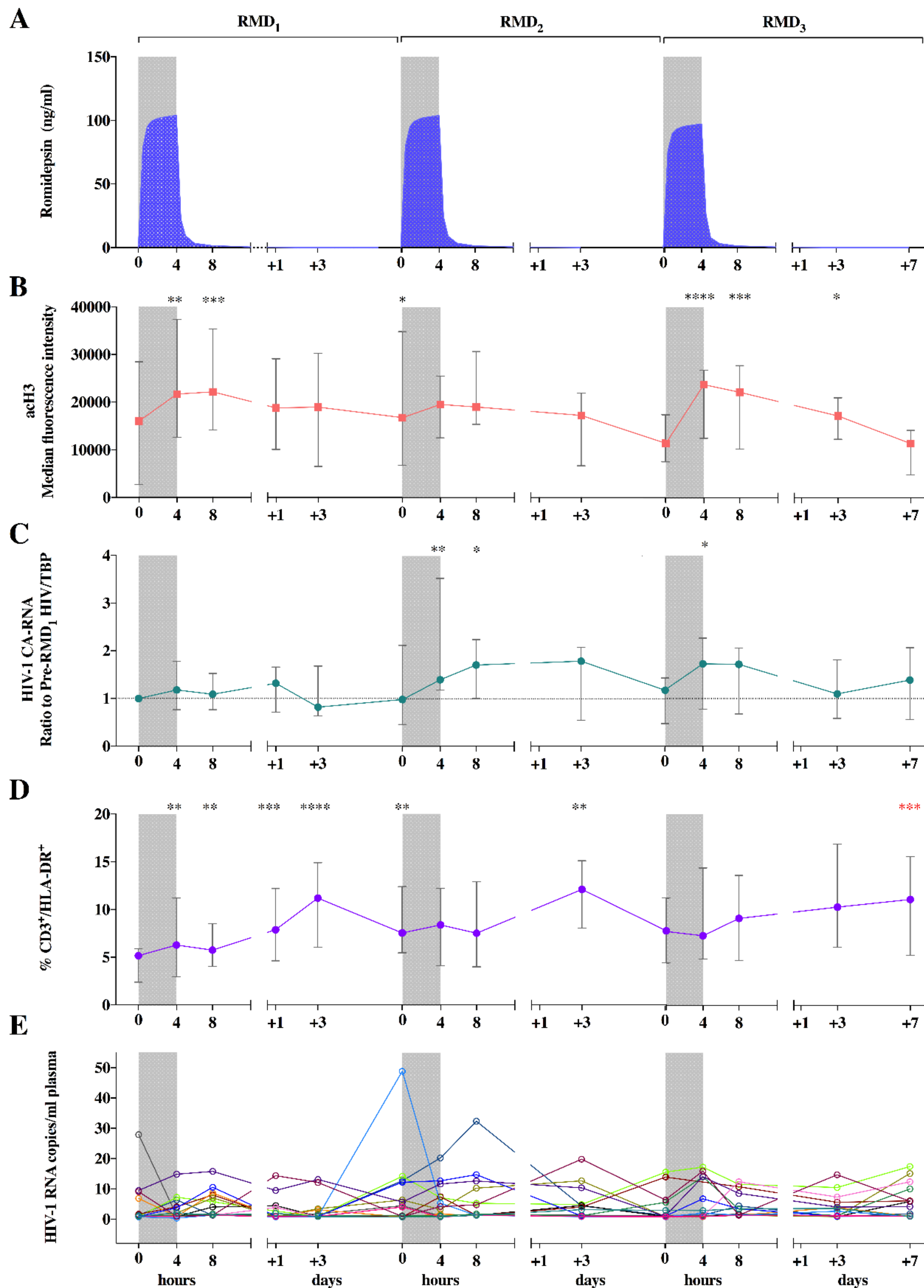

Fig. 2. Pharmacokinetic and pharmacodynamic effects of RMD (A) Simulated RMD plasma concentrations. (B) Levels of histone H3 acetylation in peripheral lymphocytes. (C) Viral transcription levels expressed as changes from pre-RMD_1 levels of cell-associated HIV-1 RNA in peripheral $CD4^+$ T-cells. (D) Levels of T-cell activation ($CD3^+$/$HLA\text{-}DR^+$ cells). (E) individual determinations of pVL. Median of frequencies and IQR (error bars) are represented. Wilcoxon signed-rank *p* values compare each represented time point with the corresponding values preceding each RMD administration. *, p<0.05. **, p<0.01. ***, p<0.001 and ****, p<0.0001. The *p* value resulting from the comparison between the value at day of RMD_1 and 7 days after RMD_3 is shown in red.

MVA.HIVconsv immunogenicity

Total HIV-1 and HIVconsv-specific T cells were assessed *ex vivo* by an IFN-γ-detecting enzyme-linked immunoabsorbent spot (ELISPOT) assay using 6 peptide pools covering the HIVconsv immunogen sequence (P1-P6) at 6 time points: week 0 (day of MVA_1), 1, 3 (day of RMD_1), 9 (day of MVA_2), 10, and 13. A total of 90 samples were obtained, of which 3 (3%) were censored due to low positive controls and/or high background. All 15 participants (100%) showed an absolute increase in HIVconsv-specific IFN-γ-producing T cells during the study, either after MVA_1 (Wilcoxon signed-rank, p=0.0007) or after MVA_2 (Wilcoxon signed-rank, p=0.0017) (Fig. 3B). Median (range) total frequencies of HIVconsv-specific T cells reached 1,965 (530-6,901) spot-forming cells (SFC)/10^6 PBMC at the peak immunogenicity time point, which represented an absolute increase of 1,600 (300 – 6,621) SFC/10^6 PBMC from baseline (Wilcoxon signed-rank, p <0.0001).

Over the intervention phase, participants responded to a median (range) of 5 (2-6) peptide pools (Fig S4). To map the maximum vaccine-induced breadth at peak immunogenicity time point (weeks 10-13), *in vitro* expanded T cells responding towards individual OLPs covering the HIVconsv immunogen were assessed. A median (range) of 8 (3-16) IFN-γ-producing responses to individual OLPs were found, with a dominance in Pol-specific T cells, consistent with the immunogen composition (Fig. 3C).

The dominance of HIVconsv-specific responses was calculated at each time point as the percentage of HIVconsv-specific T-cell frequencies divided by the total HIV-1 proteome-specific T-cell frequencies. At the moment of HIV diagnoses, HIVconsv responses were subdominant (less than 10% being HIVconsv-specfic) and peaked after the CM vaccination reaching a median (range) of 58% (7%–100%) of the total anti-HIV-1 T-cell responses (BCN01 parental study) (19). In BCN02, over 2 years from the last HIVconsv vaccination, the increase in the frequency of HIVconsv-specific T-cell responses after MVA_1 or MVA_2 further shifted the patterns of T-cell immuno-dominance towards HIVconsv with a median (range) of 85% (54%-100%) of the total anti-HIV-1 T-cell responses at peak immunogenicity time point (Fig. 3D).

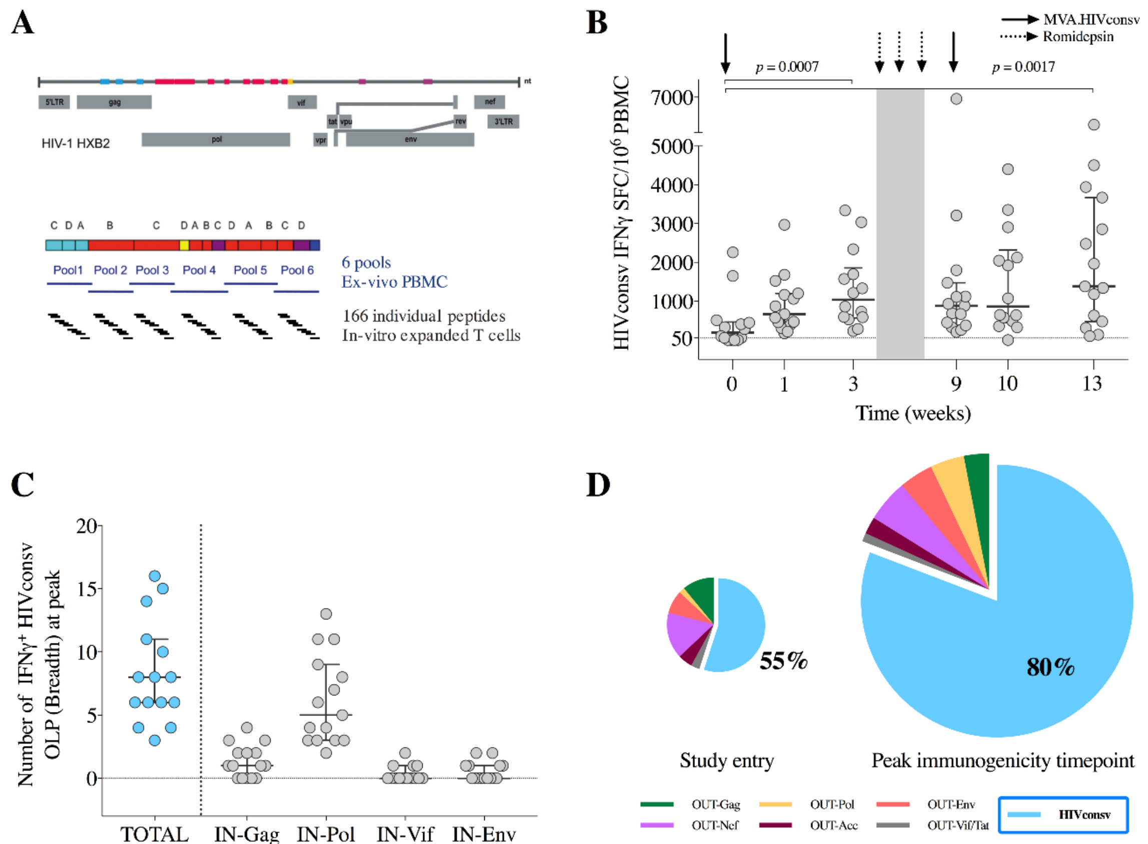

Fig. 3. Vaccine immunogenicity (A) Schematic representation of the selected regions in the HIV-1 proteome from different clades included in the HIVconsv immunogen and distribution of 6 peptide pools (P1-P6) and individual overlapping 15-mer peptides (OLP) used in the IFN-γ ELISPOT assays. (B) Magnitude (sum of SFU/10^6 PBMC to pools P1-P6) of vaccine-induced responses over the BCN02 study. Horizontal and error bars represent median and IQR, respectively, and *p*-values correspond to comparisons between the indicated time points using the Wilcoxon signed-rank test. (C) Breadth of vaccine-elicited responses towards individual OLP included in the indicated HIVconsv regions. Horizontal and error bars represent median and IQR, respectively. (D) Average distribution of total HIV-1 T-cells according to their specificity at the indicated time points. HIVconsv-specific responses are shown in blue. Pie charts are scaled according to the total frequencies of responses.

Effects on the HIV-1 reservoir

All participants had detectable viral reservoirs, as measured by total $CD4^+$ T cell-associated HIV-1 DNA, throughout the study. Results from 2 samples out of a total of 60 were considered invalid and were censored. At BCN02 study entry, median (range) reservoir size was of 140 (17-752) HIV-1 DNA copies/10^6 $CD4^+$ T-cells (Fig. 4). Proviral DNA showed a tendency to further decrease from baseline to week 17 (Wilcoxon signed-rank, p=0.0599, Fig. 4) to median (range) levels of 120 (11-680) copies / 10^6 CD4+ T cells.

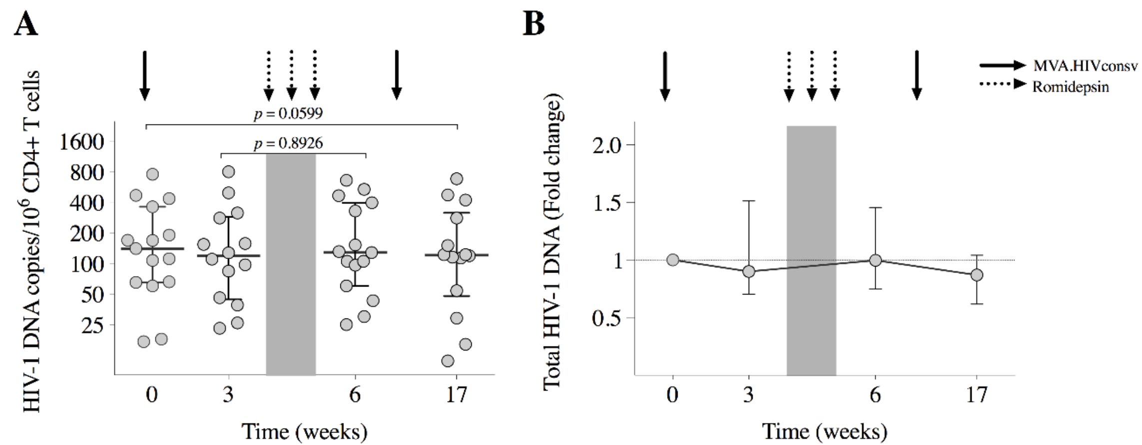

Fig. 4. Viral reservoir. (A) Total HIV-1 DNA copies/10^6 $CD4^+$ T cells for each participant are shown at study entry (week 0), week 3 (day of RMD_1) and week 6 (1 week after RMD_3) and at week 17 (8 weeks after MVA_2). Horizontal and error bars represent median and IQR, respectively, and *p*-values correspond to comparisons between the indicated time points using the Wilcoxon signed-rank test. (B) Changes in proviral DNA throughout the study with respect to baseline (week 0), which is considered to be 1.

Monitored antiretroviral pause (MAP)

Participants undergoing MAP were monitored weekly for the first 12 weeks and every 2 weeks thereafter for a maximum of 32 weeks (MAP_{0-32}). Criteria for ART resumption included pVL over 2,000 copies/ml in two consecutive determinations, CD4 cell counts decrease over 50% and/or below 500 cells/mm^3 and/or development of clinical symptoms suggestive of an acute retroviral syndrome.

All participants rebounded (detectable pVL over 20 copies/ml) during MAP (Fig. 5A). Median (range) time to first detectable pVL was 13 (7 – 35) days with median (range) of 1st pVL of 122 (28-3,410) copies/ml. Ten participants resumed ART before MAP_{12} (MAP-NC for MAP-*Non-controllers*) qith median (range) time to resume ART was at 28 (16-59) days. All MAP-NC resumed ART due to the viral load criteria, with median (range) pVL of 19,250 (2,900 – 179,000) copies/ml at the moment of ART resumption (Fig. 5B). None of the participants resumed ART due to immune or clinical criteria. A 'late-rebounder' presented the first detectable pVL five weeks after ART interruption (MAP_5 with 59 copies/ml) and was able to maintain viral load below 2,000 for 3 more weeks, resuming ART at MAP_8. In addition to the 'late-rebounder', 3 (23%) other participants remained off ART with sustained pVL <2,000 copies/ml for a total of 32 weeks (MAP-C, from MAP-*Controllers*). All participants who restarted ART reached viral re-suppression within 6 months. No evidence of emergence of drug resistance was detected.

To assess re-seeding of the viral reservoir during the MAP, total HIV-1 DNA was measured at MAP_0 (n=13), on the day of ART resumption (n=10) and 6 months after for the 10 MAP-NC (n=8 available), and at MAP_{32} for the three MAP-C. We did not observe any significant change in total HIV-1 DNA during MAP in participants with early ART resumption (Wilcoxon signed-rank, p=0.5759 for MAP-NC) and, noteworthy, nor in the three participants with sustained low-level viremia for 32 weeks. Moreover, one of the three MAP-C showed a 2-fold reduction in the HIV-1 DNA (from 34 at MAP_0 to 16 copies/10^6 CD4 cells at MAP_{32}, Fig. 5C). This was the only participant carrying HLA alleles associated with natural HIV control (HLA-B*27:01/HLA-B*51:01) [35, 36].

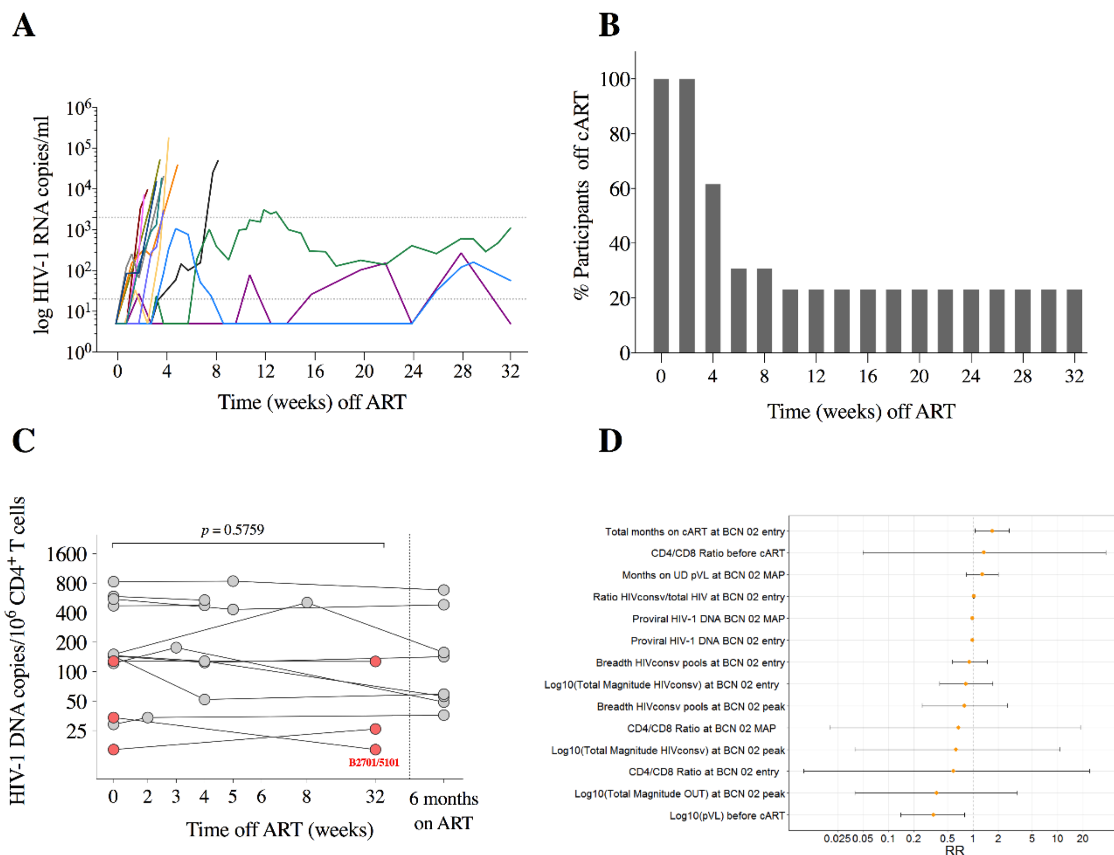

Fig. 5. Monitored antiretroviral pause. (A) Individual pVL during MAP is shown for each participant in different colours (n=13) Lines are interrupted on day of ART resumption. Dotted lines represent detection limit and threshold for ART resumption (20 and 2,000 copies/ml, respectively). (**B**) Proportion of individuals remaining off ART during MAP. (**C**) Total HIV-1 DNA copies/10^6 CD4⁺ T cells are shown for each participant at MAP initiation, at day of ART ressumption, and 24 weeks after ART. *P*-value corresponds to comparison between the indicated time points using the Wilcoxon signed-rank test. Individuals with sustained low-level viremia over 32 weeks are shown in red. (**D**) Estimated relative risks and 95% confidence intervals for the durable control of pVL during MAP obtained from univariate logbinomial regression models.

Factors influencing viral rebound kinetics

Table S1 shows the absolute frequencies of variables explored during MAP to explain the binary outcome defined as MAP-NC vs MAP-C. The estimated relative risks obtained from the log-binomial models for different covariates analyzed are shown in Fig. 5D.

Univariate log-binomial regression models used to detect factors associated with virologic control during MAP revealed that pVL before ART initiation (pre-ART pVL) was the only factor statistically significantly associated with control of viral rebound after ART interruption. For each log increase on the pre-ART pVL, the probability of becoming a controller decreased by 66% (RR 0.34; 95% CI 0.14, 0.79). Interestingly, albeit not statistically significant in the univariate models, the 3 MAP-C had lower reservoir levels (MAP-C had 16, 54 and 122 copies/10^6 $CD4^+$ T cells at week 17) –consistent with lower pre-ART pVL– and showed high frequencies of vaccine-induced HIVconsv-specific responses (>700 HIVconsv-specific SFC/106 PBMCs and >75% of HIVconsv dominance over the total anti-HIV-1-specific T-cell responses at peak immunogenicity time point, Fig. S5).

Discussion

In this single-arm, open-label, phase I, proof-of-concept trial performed in HIV-1-infected ART-suppressed individuals treated during acute/recent HIV-1 infection, we show that combination of the HIVconsv vaccines with RMD as a latency reversing agent was safe, highly immunogenic, and induced bursts of viral transcription. The combined intervention resulted in a tendency towards a reduction in proviral DNA levels and was followed by a prolonged viremic control in 23% of participants after ART interruption without evidence of reservoir re-seeding.

Early-treated individuals typically show less immune exhaustion [37] and reduced frequency of immune-escaped viral variants compared to individuals treated during later stages of HIV-1 infection [38], offering a potentially favorable setting to induce a protective immune response upon therapeutic vaccinations. In the parental BCN01 trial, a prime/boost vaccine regimen with ChAdV63.HIVconsv and MVA.HIVconsv induced high frequencies of T cells with high *in vitro* suppressive capacity that markedly shifted the focus of the $CD8^+$ CTL response towards HIVconsv sequences that are subdominant during natural infection [19]. In the BCN02 trial, after 3 years of viral suppression and 2 years since the last vaccination in BCN01, booster MVA.HIVconsv vaccinations were still

immunogenic and further increased breadth, magnitude and immunodominance of CTL responses towards HIVconsv sequences.

Along with a strong vaccine-elicited CTL activity ("kill"), the ability to simultaneously induce reactivation of the viral reservoir ("kick") is a critical feature for the success of the kick&kill strategy [23]. A 3-dose regimen of weekly RMD at 5 mg/m^2 BSA was selected based on results from previous trials [30, 31]. Consistently, we observed a direct *in vivo* effect of RMD in histone 3 acetylation upon each RMD dose, which was followed by changes in cell-associated HIV-1 RNA levels. Conversely, a placebo-controlled dose-escalating trial (ACTG 5315) testing 3 RMD doses (5 mg/m^2 BSA) administered every two weeks in chronically suppressed individuals, did not show changes in viral transcription [39]. The weekly administration regimen the intense sampling after each RMD dose in our study allowed us to detect increases in CA HIV-1 RNA above 2-fold in 80% of individuals at any time point after RMD administrations. These changes were followed by consistent cumulative increases in T cell activation markers, which remarkably, were maintained one week after RMD_3, likely reflecting a direct additive effect of weekly RMD dosing and an effective induction of viral transcription. Noteworthy, changes above 2.1-fold in CA-RNA have been estimated to occur in less than 5% of repeated measurements in an individual [31].

Despite the induction of viral transcription, the kinetics of plasma HIV-1 RNA followed an unclear pattern, similar to previous studies showing variable changes in plasma viremia following LRA administration [31, 40, 41]. This variability might reflect suboptimal potency of the agents tested so far and/or the reactivation of predominantly defective proviruses. In the context of the current study, elimination of reactivated cells by vaccine-elicited T cells may have additionally blunted quantification of plasma viremia.

A critical objective of the use of LRA in a kick&kill strategy is to mobilize and ultimately reduce the viral reservoir. Our findings showed that, despite robust immunogenicity of HIVconsv vaccines and at least partial reactivation of the viral reservoir induced by RMD, the net effect on the proviral DNA levels was modest. All participants had detectable levels of HIV-1 DNA at the time of treatment interruption although a tendency towards a decrease by 19.3% from baseline to week 17 (Wilcoxon signed-rank, p=0.0599) was detected. Conversely, mean 39.7% decrease in reservoir size was observed in the REDUC trial [31]. This discrepancy between REDUC and BCN02 results may be explained by the inclusion of early-treated individuals in the latter study, in which basal levels of proviral DNA were substantially lower, challenging the quantification of the effects of the intervention on HIV-1 DNA levels. We acknowledge that the traltation of

HIV-1 protein expression into antigen presentation - even in case of defective proviruses (1) - upon LRA reactivation is poorly understood. Likewise, the ability of LRA-induced HIV-1 protein expression to effectively induce recognition and killing by $CD8^+$ T cells remains to be fully elucidated [42] and therefore, the potential effects of further RMD administrations on the viral reservoir are to be determined. A potential significant toxicity –suggested in *in vitro* assays [43]- on vaccine-induced T cells might also have limited our capability to observe a further reduction in the reservoir size in our study. This hypothesis is consistent with the fact that HIVconsv vaccines induced higher levels of activated T cells compared to Vacc-4x vaccination in the REDUC trial. Nevertheless, the potential toxicity of RMD *in vivo*, its relationship with RMD exposure and, ultimately, whether vaccine-induced T cells were able to sensor and remove infected cells in response to HIV-1 reactivation remains to be determined.

To attain a functional cure, a persistent immune-mediated control of residual HIV-1 might be as relevant as achieving an absolute reduction on the proviral DNA levels. In this regard, the three BCN02 MAP-C, were among the subjects with both lower viral reservoir levels at MAP and higher vaccine-induced responses. In our study, having lower pre-ART pVL was the only outstanding marker associated with viral control during MAP, which correlates with the size of the viral reservoir after ART suppression. These relationships are consistent with previous studies suggesting a role of a low viral reservoir on ATI outcomes. Furthermore, after ART discontinuation, MAP-C did not show an initial burst in pVL followed by a post-peak control as described in several post treatment controllers (PTC) [7, 44]. Collectively, the findings from this and other studies suggest that a small reservoir size, resulting from early ART or another intervention, is essential to achieve sustained post-intervention control [45] but also, that a potent vaccine-induced immune pressure might contribute to prevent a peak burst of viremia and maintenan suppressed viremia for a substantial period of time. This control, mediated by immune pressure, is supported by the absence of re-seeding of the viral reservoir in the BCN02 MAP-C, in contrast to what other ATI trials have reported [46, 47].

The interpretation of the outcome of kick&kill studies may be confounded by individuals controlling HIV rebound after treatment interruption without the need for a prior therapeutic intervention. The prevalence and mechanisms driving PTC in natural HIV infection are not well understood. A recent metanalysis (CHAMP study) including 14 interruption trials estimated a 13% rate of PTC among early-treated individuals [44]. Importantly, and in contrast to the three BCN02 MAP-C who did not show a transient

higher burst of viremia, 32% of the 61 PTCs analyzed in the CHAMP metanalysis had transient peaks of viremia ranging from 1,000 to over 10,000 copies/ml within the first 24 weeks after treatment interruption. Thus, in addition to the different behavior of the 3 MAP-C (23%) with respect to the PTC, according to this metanalysis, the BCN02 trial may have missed over additional MAP-C due to the conservative ART resumption criteria (two consecutive pVL over 2,000 copies/ml).

The safety and tolerability profiles of MVA.HIVconsv and RMD were similar to those reported in previous studies [18, 19, 31]. However, there was a SAE in one participant. This case highlights the need for planning intensive monitoring in this kind of pilot trials, even if not powered to detect low-frequency AE, and points towards the need for a trade-off between the number of participants potentially put at risk in well-powered controlled-trials and for caution with the use of uncompletely characterized agents in large numbers of individuals. Given that natural PTC rates are considered to be up to 13% in early-treated individuals, powering trials to show viral control efficacy after an ATI becomes challenging [48]. Despite frequent clinical monitoring for pVL and access to psychological support, protocol violation during MAP occurred in one individual probably due to a suboptimally managed anxiety secondary to the antiretroviral interruption. This case warrants a closer psychological management in longer term ATIs.

We fully acknowledge the limitations of the small sample size and lack of a control arm in the present study. Therefore, we interpret these results with caution and regard this study only as hypothesis-generating trial for future interventions. BCN02 eligibility was restricted to vaccinated participants in the parental open-label BCN01 trial. This intrinsic restriction limited the sample size to a small number of previously vaccinated individuals and precluded the inclusion of a control arm. At the time of trial design, interventional trials including an ATI were typically small and included very conservative ART resumption criteria [46, 49]. Furthermore, ATI acceptability by participants, risks of HIV-1 transmission to others in the absence of available PrEP, and potential viral re-seeding upon treatment interruption were of special concern in early-treated individuals, who had limited viral reservoirs both in size and diversity [38].

Altogether, the results from this pilot study suggest a potential role for kick&kill strategies in inducing durable immune-mediated HIV-1 control in a proportion of early-treated individuals. In view of these results, future controlled studies to identify the mechanisms underlying sustained HIV-1 suppression are warranted.

Acknowledgements

The authors thank i2e3 for providing medical writing support. We especially thank Agueda Fernandez, Rafaela Ayen, Lucía Gómez and Carmen Ligero for their technical assistance. Special thanks to all the volunteers participating in this study for their perseverance and dedication. ***Funding**:* BCN02 study was funded by the ISCIII PI15/01188 grant, the HIVACAT Catalan research program for an HIV vaccine and the Fundació Gloria Soler. Some sub-analyses were partly funded by the European Union's Horizon 2020 research and innovation program under grant agreement 681137-EAVI2020 and by NIH grant P01-AI131568. The GMP manufacture of the MVA.HIVconsv vaccines was jointly funded by the Medical Research Council (MRC) UK and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreements (G0701669). Romidepsin was kindly provided by Celgene Ltd. BM held a post-doctoral fellowship grant from ISCIII (JR 13/00024) from 2014–16. CMa held a post-doctoral research grant (PERIS- 2016/2020) from the 'Departament de Salut de la Generalitat de Catalunya', Barcelona, Spain during the conduct of the study. SML held a PhD grant from DGR (2013FI_B 00275) from 2013–16. JMM received a 80:20 research grant from IDIBAPS, Barcelona, Spain, during 2017–19. GG, KL and YA have been partially supported by Grant MTM2015-64465-C2-1-R from Ministerio de Economía y Competitividad. Author contributions: BM, JM, BC and CB conceived and designed the study. CMa, TH, JMM, JMP, AMB, PC, MM, MV contributed to the study design. CM, CMa, IR, JT, ACa, MRU, MCP contributed to data management. BM, MRU, MCP, ML, SML, ALL, JM, PC, ACa, CM, SC, AMB and CMa performed the experiments. JM, BM, MCP, MV, YA, GG and KL undertook the statistical analysis. AC and TH contributed with reagents/materials/analysis tools. BM, JM and CB drafted the manuscript. TH, MRU, CMa, MCP, SML, JMP, JMM, MV and BC participated in study analyses and revised the manuscript critically for important intellectual content. All authors reviewed and approved the final version of the manuscript. Competing interests: BM is a consultant for AELIX THERAPEUTICS, S.L., outside the submitted work. JMM reports personal fees from Abbvie, Angelini, Contrafect, Genentech, Gilead, Jansen, Medtronic, MSD, Pfizer, ViiV Healthcare, outside the submitted work. TH has a patent US 7981430B2 issued. CB is founder, CSO and share holder of AELIX THERAPEUTIC, S.L. BC is founder, consultant and share holder of AELIX THERAPEUTICS, S.L. All other authors declare that they have no competing interests.

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Supplementary material

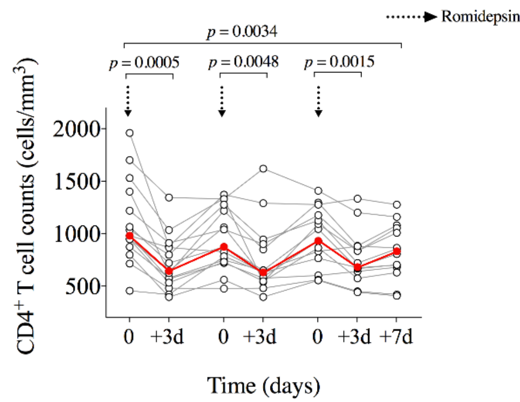

Fig. S1. Evolution of CD4⁺ T cells counts during the 3 RMD infusions is shown for all 15 participants. Median is shown with a red line. *P*-values correspond to comparisons between the indicated time points using the Wilcoxon signed-rank test.

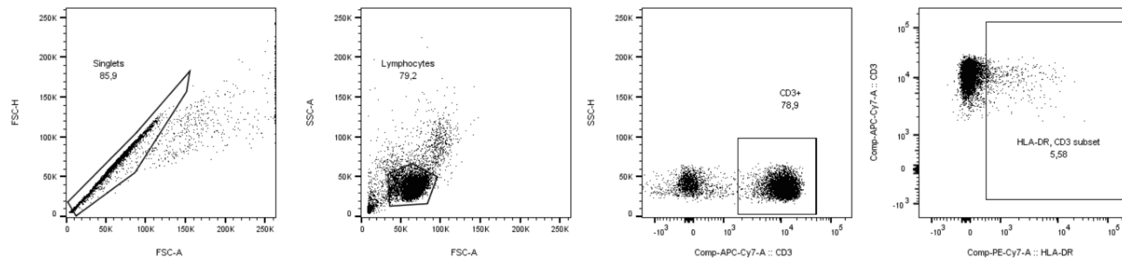

Fig. S2. Gating strategy for T-cell activation analysis based on HLA-DR expression in CD3 T cells: Singlet gate (FSC-H vs. FSC-A) -> Lymphocyte gate (SSC-A vs. FSC-A) -> CD3⁺ T cells (SSC-H vs. CD3) -> CD3⁺ HLA-DR⁺ (CD3 HLA-DR).

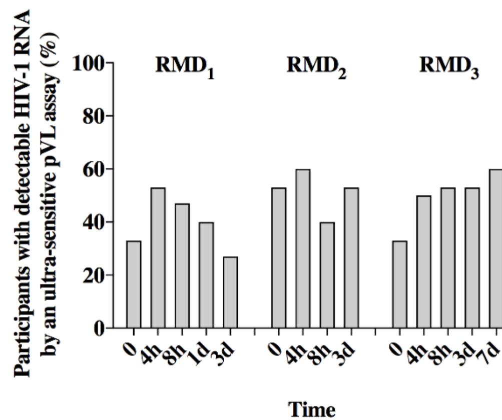

Fig. S3. Proportion of participants with detectable HIV-1 RNA during the three RMD administrations by an ultra-sensitive viral load assay using 4-8 ml plasma.

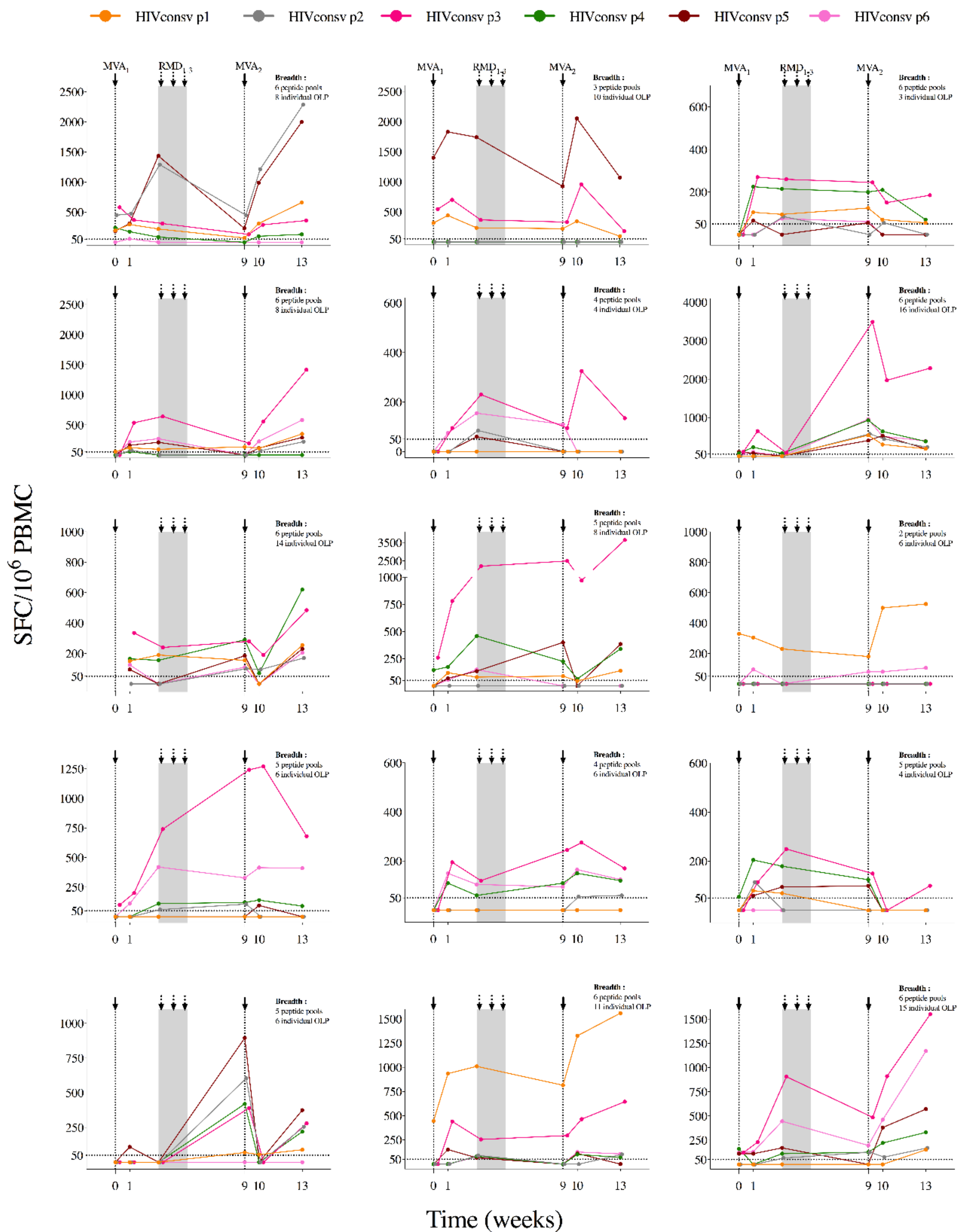

Fig. S4. Responses to individual HIVconsv peptide pools (P1-P6) for each participant (n=15) are shown. The response breadth is calculated as the number of peptide pools giving a positive response during the trial (out of 6 pools) in the *ex-vivo* ELISPOT assay and the number of individual HIVconsv OLP giving a positive response in the ELISPOT using *in-vitro* expanded PBMC at peak immunogenicity time point is indicated.

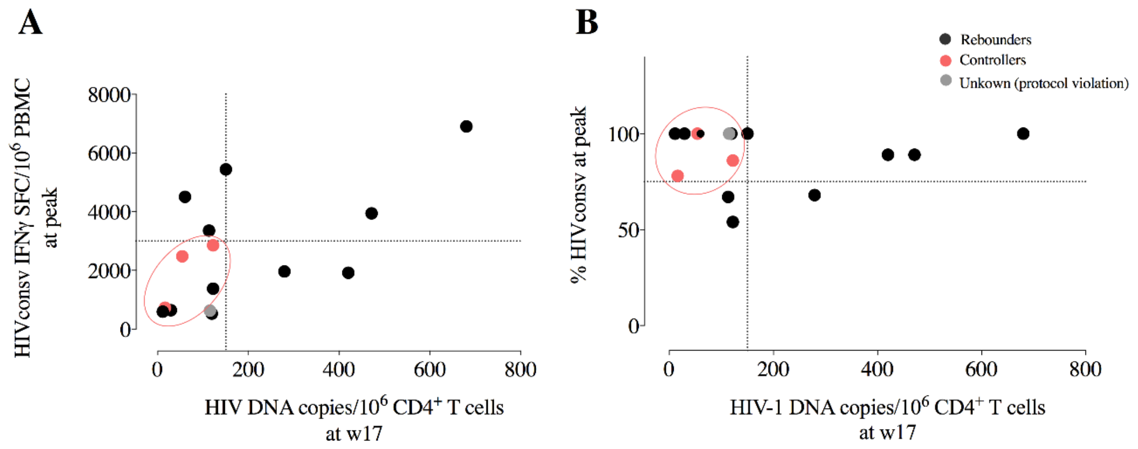

Fig. S5. Distribution of proviral DNA levels after RMD and vaccine elicited responses at peak immunogenicity time point among the 15 participants.

Table S1. Summary of continuous variables included in the log-binomial models

Variable	MAP - *Non Controllers* (n=10)				MAP - *Controllers* (n=3)			
	Med	IQR	Min	Max	Med	IQR	Min	Max
Demographics								
Age	42	38-45	33	48	32	31-36	30	40
Before any ART initiation								
Days from HIV-1 to ART	82	67-107	32	118	112	70-138	28	164
log_{10} (pVL) before any ART	5.02	4.88-5.16	4.26	5.48	3.35	3.28-4.59	3.20	5.82
CD4 absolute before any ART	453	382-560	299	785	631	608.5-633	586	635
CD4/CD8 ratio before any ART	0.56	0.45-0.69	0.44	1.26	0.56	0.42-0.85	0.27	1.14
At BCN02 entry								
CD4 absolute	728	533-1331	416	1408	657	652.5-814	648	971
CD4/CD8 ratio	1.37	1.22-1.47	1.00	1.93	1.33	1.15-1.54	0.97	1.74
Total months on ART	38.88	37.56-40.20	36.36	41.04	41.64	39.30-42	36.96	42.36
Months on UD pVL	35.52	35.40-35.88	31.32	40.32	36.36	36.18-39	36	41.64
At MAP								
CD4 absolute	735	484-1075	468	1269	854	675-902	496	950
CD4/CD8 ratio	1.30	1.25-1.48	0.87	1.75	1.52	1.14-1.57	0.76	1.61
Total months on ART	41.88	44.04-46.32	41.88	46.80	46.80	44.88-47.28	42.96	47.76
Months on UD pVL	41.52	40.92-43.68	37.80	45.60	42.48	42.24-44.34	42	46.20
Vaccine immunogenicity								
HIVconsv magnitude								
At BCN 02 entry	160	0-287	0	2640	0	0-655	0	1310
At BCN 02 peak	1965	1380-3940	530	6901	2480	1605-2668	730	2855
HIVconsv pools breadth								
At BCN 02 entry	1	0-2	0	2	0	0-1	0	2
At BCN 02 peak	4	4-6	3	6	6	5.5-6	5	6
HIVconsv immunodominance								
At BCN 02 entry	6	0-8	0	37	0	0-38	0	76
At BCN 02 peak	89	68-100	54	100	86	82-93	78	100
Responses OUTside HIVconsv								
At BCN 02 entry	4525	3088-5385	1635	8945	3328	1872-3428	415	3528
At BCN 02 peak	705	405-1150	130	5385	530	325-925	120	1320
Viral reservoir								
Week 0 BCN 02	190	107-434	18	752	65	62.5-116.5	60	168
Week 3 BCN 02	157	110-494	26	892	46	34.5-100	23	154
Week 6 BCN 02	131	105-464	60	656	43	36.5-185.5	30	328
Week 17 BCN 02	144	119-420	29	680	54	35-88	16	122
RMD-PK								
AUC_1	392.9	386-427.1	314	439.5	473.5	432.5-548.9	391.5	624.3

ART: antiretroviral treatment; UD pVL: undetectable plasma viral load; MAP: Monitored antiretroviral pause; RMD: Romidepsin

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This declaration concerns the following article/manuscript:

Title: HIVconsv vaccines and romidepsin in early-treated HIV-1-infected individuals: Safety, immunogenicity and effect on the viral reservoir (study BCN02).
Authors: Mothe B, Rosas-Umbert M, Coll P, Manzardo C, Puertas MC, Morón-López S, Llano A, Miranda C, Cedeño S, López M, Alarcón Soto Y, Gómez Melis G, Langohr K, Barriocanal AM, Toro J, Ruiz I, Rovira C, Carrillo A, Meulbroek M, Crook A, G. Wee E, Miró JM, Clotet B, Valle M, Martinez-Picado J, Hanke T, Brander C and Moltó J, for the BCN02 study investigators.

The article/manuscript is: ☐ Published ☐ Accepted ■ Submitted ☐ In preparation

Submitted to Science Translational Medicine

The phD student has contributed to the elements of this article/manuscript as follows:

A. No or little contribution
B. Has contributed (10-30%)
C. Has contributed considerably (40-60%)
D. Has done most of the work (70-90%)
E. Has essentially done all the work

Formulation/identification of the scientific problem	B
Planning the experiments and methodology design and development	C
Involvement in the experimental work/data collection	B
Interpretations of the results	B
Writing the first draft of the manuscript	B
Finalization of the manuscript and submission	B

The work presented in this Chapter was a project granted by FIS applied by Drs Mothe and Moltó. The phD student contributed to the grant application. The phD student performed relevant background literature search. The phD student contributed to the design of the lab analytical plan of the project and was responsible of managing the samples from different clinical sites that participated in the trial. As for experimental work, contributed to the vaccine immunogenicity analysis and performed all flow cytometry experiments described in the paper.

Dr Christian Brander (director) Dr. Beatriz Mothe Pujadas (director)

CHAPTER IV

In vivo effects of romidepsin on T cell activation, apoptosis and function in the BCN02 Kick&kill clinical trial

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(in preparation)

Abstract

Romidepsin is a well-characterized histone deacetylase inhibitor (HDACi) approved for the treatment of cutaneous T-cell lymphoma (CTCL). *In vitro* and *in vivo* studies have demonstrated that it is able to induce HIV gene expression in latently infected $CD4^+$ T cells from HIV^+ individuals on cART. However, *in vitro* experiments suggested that romidepsin could also impair T cell functionality, particularly in activated T cells. Thus, the use of romidepsin in HIV kick&kill strategies that aim to enhance the immune system towards eliminating infected cells after inducing viral reactivation, may be restricted. In order to address whether the *in vitro* observations could be translated into *in vivo*, we determined the effects of romidepsin on the total and HIV-specific T cell populations in longitudinal samples from the BCN02 therapeutic vaccine clinical trial (NCT02616874). BCN02 was a proof-of concept study in 15 early treated $HIV\text{-}1^+$ individuals that combined MVA.HIVconsv vaccination with 3 weekly infusions of romidepsin given as a latency-reversing-agent (LRA). Our results showed that romidepsin induced a transient increase in the frequency of apoptotic T cells and an enhanced activation of vaccine-induced T cells. Although romidepsin reduced the number of HIVconsv-specific T cells secreting multiple cytokines induced by vaccination, viral suppressive capacity of $CD8^+$ T cells was preserved over the romidepsin treatment. These observations may have important implications for the design of effective kick&kill strategies for the cure of HIV.

Introduction

Current antiretroviral therapy (ART) effectively suprresses HIV replication in plasma but is not able to completely eliminate the virus from infected individuals. Therefore, antiretroviral cessation results in a rebound of plasma viremia within weeks in essentially all individuals [1]. Rapid viral rebound after treatment interruption is due to the existence of a latent viral reservoir and the inability of the immune system to effectively contain viral rebound. To date, numerous strategies have been proposed to achieve a functional cure or virus eradication, including early ART initiation, ART intensification [2–6], passive administration of antibodies [7], therapeutic vaccines [8–13] or gene therapy [14,15], among others.

Kick&kill strategies propose to use latency reversing agents (LRA) to induce the transcription of HIV genes from latently infected cells and render these cells susceptible to vaccine-induced, activated, virus-specific cytolytic T lymphocytes (CTL). The interest in LRA able to reactivate the latent virus has increased over the past decade, with histone

deacetylation inhibitors (HDACi) being one of the best characterized agents both, in vitro and in vivo. The effect of HDACi on inhibiting histone deacetylation results in a higher degree of acetylated histones, causing an increased gene transcription. In ex vivo isolated cells from ART suppressed HIV infected individuals, exposure to HDACi results therefore in reactivation of the integrated HIV genes and leads to viral protein expression by latently infected cells [16–18]. Some HDACi, such as vorinostat (SAHA), panobinostat and romidepsin have also been tested for their in vivo activity to reverse HIV-1 latency [3-5]. Romidepsin (RMD), a cyclic depsipeptide naturally produced by *Chromobacterium violaceum*, is a pan-HDACi that inhibits class I HDACs. RMD was clinically developed as an anti-cancer drug and is approved for the treatment of cutaneous T-cell lymphoma [19]. Furthermore, RMD has been shown to induce HIV gene expression in latently infected cells in vitro [20,21] and in vivo, when administrated alone [22] or in combination with the therapeutic vaccine Vacc-4x [23] in chronically ART suppressed HIV infected individuals. Although it was first thought that reactivation of the virus itself would lead to robust immune activation and control of the rebounding virus, it is generally accepted that further stimulation of the immune effector response by a therapeutic vaccine and/or an immune checkpoint inhibitor, may be needed in order to efficiently eliminate infected cells after LRA exposure [24]. Therefore, different immunotherapies are being investigated together with LRAs to test their combined effect, especially combination treatments that include T-cell vaccines.

The proof-and-concept BCN02 trial evaluated a kick&kill strategy that combined the MVA.HIVconsv T-cell vaccine with the HDACi romidepsin in a cohort of early-treated, HIV-1- infected individuals. Fifteen individuals were recruited and were immunized with two doses of MVA.HIVconsv (MVA1-2) before and after three weekly-doses of RMD (RMD1-2-3) followed by a monitored antiretroviral pause (MAP) for a period of 32 weeks (NCT02616874). The combined strategy was proven to be safe and vaccination was highly immunogenic. RMD treatment resulted in marked increases in histone acetylation and cell-associated HIV-1 RNA levels, compatible with induction of viral transcription. However, the ultimate reduction of the viral reservoir was overall minimal in BCN02 trial (Mothe, Rosas et al, submitted). Importantly, for a successful purge effect in the viral reservoir, it will be critical that the LRA used in such strategies does not have any detrimental effects on the vaccine-induced immune cells [25,26]. Here, we assessed the in vivo impact of 3 weekly RMD doses on total and vaccine-induced T cells in longitudinal samples from BCN02 trial (Fig. 1).

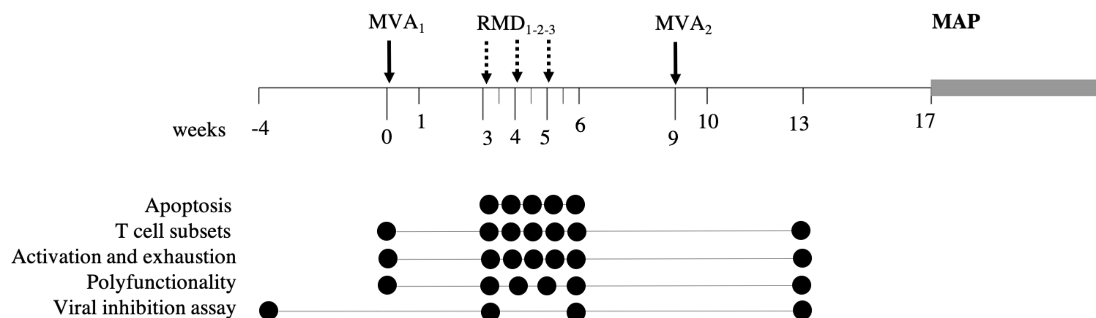


Figure 1: Study design. BCN02 study was a single arm, open label, proof-of-concept study to address safety and effect on the viral reservoir of a kick&kill strategy combining MVA.HIVconsv vaccines with the HDACi romidepsin. Timepoints used for the analyses presented here are indicated for each assay by filled circles.

Material and Methods

Study and samples

The BCN02 clinical trial (NCT02616874) was a phase I, open-label, single-arm, multicenter, single-country study (Mothe, Rosas-Umbert et al, submitted). The study was approved by the institutional ethical review board of the participating institutions (Reference Nr AC-15-108-R) and by the Spanish Regulatory Authorities (EudraCT 2015-002300-84); and was conducted in accordance to the principles of the Helsinki Declaration and local personal data protection law (LOPD 15/1999). Fifteen participants were immunized with MVA.HIVconsv (MVA_1, $2x10^8$ pfu), followed by three weekly-doses of romidepsin (RMD_{1-2-3}, 5 mg/m^2 BSA) and by a second MVA.HIVconsv booster vaccination (MVA_2, $2x10^8$ pfu) before undergoing a monitored antiretroviral pause (MAP) for a maximum of 32 weeks. Cryopreserved peripheral blood mononuclear cells (PBMC), plasma and serum samples were stored before, at the end and after 8h, 24h (only for RMD_1, and 3 and 7 days after all RMD doses for immunological and virological studies.

Flow Cytometry

Apoptosis measurement

Cell viability was detected using a “Pacific Blue™ Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend). Annexin V stains phosphatidyl serine, which is exposed on the cell surface of apoptotic cells and 7AAD binds to DNA. Therefore, Annexin V- /7-AAD- represent viable cells, Annexin V^+ / 7-AAD^- cells are in the early stages of apoptosis, while Annexin V^+/7-AAD^+ cells are in late apoptotic phase, and necrotic cells are (Annexin V^-/7-AAD^+). Lineage surface markers (CD3, CD4 and CD8) and activation markers (HLA-DR, CD25 and CD69) were included to the staining.

Briefly, 1,000,000 of isolated PBMC were washed in PBS and resuspend in 100 µl of surface staining solution (CD3, CD4, CD8, CD25, CD69, HLA-DR) and incubated for 20 minutes. After 2 washes with 300 µl of PBS with 1% FSC, cells were resuspended in 100 µl of Annexin V Binding Buffer with the corresponding AnnexineV and 7-AAD. After 15 minutes of incubation, 250 µl of Binding Buffer was added to each tube and acquired on a LSRII BD cytometer. The percentages of apoptotic and live cells were analyzed using FlowJo software. The gating strategy is summarized in Supplementary Figure 1.

T cell and HIV.consv-specific T cell lineage, activation and cytokine detection

Briefly, PBMCs were thawed and stimulated with CD49d and CD28 antibodies (BD) in presence/absence of peptides pools containing peptides covering the HIVconsv immunogen sequence in the presence of GolgiStop for 5 hours. Cultures were then stored overnight at 4°C until staining. Cells were stained first with a viability stain (Aqua Live/Dead Fixable Dead Cell Stain kit, Invitrogen), followed by T-cell lineage and activation markers (using CD3-APC Cy7, CD4 PECy5; CD8 PerCP, CCR7 B711, CD45RA BV785, HLA-DR BV650, PD-1 BV605, CD69 APC and CD25 PEDazzle594; BioLegend) and dump channel (using CD19-V450 for B-cells and CD14-V450 for monocytes; BioLegend) surface staining. Following the fixation and permeabilization step (Fix and Perm kit, Invitrogen), intracellular staining with antibodies for cytokines (IFN-γ A700; Invitrogen, IL-2 PE-Cy7, TNF-α FITC; BiolLegend and MIP-1β PE; RD Systems) was performed. Cells were collected on an LSRFortessa BD instrument, and analysis was performed using FlowJo 10 software. The gating strategy is summarized in Supplementary Figure 2.

Intracellular cytokine staining analysis were done applying boolean gates in FlowJo 10, subtracting unstimulated signals using Pestle v1.7 program and represented using SPICE v5.35 software (provided by the National Institute of Health, Mario Roeder, ImmunoTechnology Section, Vaccine Research Center, NIAID, NIH, Bethesda) [45].

Viral inhibition assay

$CD8^+$ T-cell mediated viral inhibition capacity was measured at different CD8:CD4 ratios. Cryopreserved PBMCs were obtained from timepoints before BCN02 intervention and $CD8^+$ cells were depleted by magnetic bead separation (MACS Milteny Biotec). $CD8^+$-depleted cells ($CD4^+$-enriched fraction) cells were stimulated with PHA (5µg/mL) in RPMI plus 10% fetal bovine serum (R10) and antibiotics (penicillin and streptomycin). After 3 days of stimulation, the CD4-enriched fraction was infected by spinoculation with HIV-1 BaL and IIIB laboratory-adapted strains at a multiplicity of infection (MOI) of 0.01 as

reported previously [12,27]. HIV-infected cells were cultured in duplicates or triplicates in R10 medium with 20IU/mL of IL-2 in 96-well round-bottomed plates, alone or together with unstimulated CD8 T cells obtained by positive magnetic bead separation the same day from an additional vial of frozen PBMC from screening (w-4), 3 weeks after MVA_1 ($postMVA_1$, week3), 1 week after RMD_3 (week 6), 4 weeks after MVA_2 ($postMVA_2$, w13) time points. Cultures at different CD8:CD4 ratios (E:T = 1:1 and 1:10) were harvested after 6 days. Cells were stained first with Aqua Live/Dead stained with surface markers (CD3 APC-H7, BD Biosciences, CD4 PerCP, BD Biosciences, and CD8 APC, BD Biosciences), then permeabilized (FIX & PERM® Cell Permeabilization Kit, ThermoFisher). Cells were then fixed (FIX & PERM® Cell Permeabilization Kit, ThermoFisher) at room temperature and finally stained with p24 antibody (KC-57-FITC; Beckman Coulter). $CD8^+$ T cell antiviral activity is expressed as % inhibition = [(fraction of $p24^+$ cells in $CD4^+$ T cells cultured alone) − (fraction of $p24^+$ cells in $CD4^+$ T cells cultured with CD8+ T cells)]/(fraction of $p24^+$ in $CD4^+$ T cells cultured alone) × 100. Cells were collected on a LSRII BD cytometer and analysis was performed using FlowJo 10 software.

Statistical analysis

GraphPad Prism version 7 for Windows (San Diego, CA) was used for statistical analysis. Mann-Whitney test and Wilcoxon matched paired test were used for unpaired and paired comparisons, respectively. Significant values were considered for p-values<0.05.

Results

Increased apoptosis after romidepsin exposure

Since RMD has been described to have a toxic effect *in vitro*, especially on activated T cells, we first measured the effect of RMD on cell viability both in total $CD8^+$ and $CD4^+$ T cells and in activated, $HLA\text{-}DR^+$ expressing cells in PBMC from BCN02 participants. Viability in total T cells was assessed by flow cytometry by Annexin V/7AAD staining before and after the three RMD doses. Increases in the number of apoptotic $CD8^+$ T cells (Annexin V^+) were detected at 24 hours after RMD_1 (Wilcoxon signed-rank, p=0.0151) and 3 days after each RMD dose (Wilcoxon signed-rank, RMD_1 p=0.0413, RMD_2 p=0.0181 and RMD_3 p=0.0833 respectively, Fig. 2A). The same pattern was observed in $CD4^+$ T cells, with apoptotic cells being significantly increased 3 days after RMD_2 and RMD_3 (Wilcoxon signed-rank, RMD_2 p=0.0067 and RMD_3 p=0.0413, respectively, Fig.

2B). Overall, increases in T cell apoptosis were more pronounced after RMD_2 and RMD_3 compared to RMD_1. One week after RMD_3 the levels of apoptotic cells in both $CD8^+$ T cells and $CD4^+$ were not restored to pre-RMD levels (p=0.0054 for $CD8^+$ and p=0.0181 for $CD4^+$), suggestive of accumulative toxic effect of RMD.

As activated T cell are believed to be particularly sensitive to RMD exposure [25], levels of apoptosis in $CD4^+$ and $CD8^+$ T cell with high or low expression of the activation marker HLA-DR were assessed. Both HLA-DR^+ and HLA-DR^- T cells showed the same profile of percentage of apoptotic cells during RMD exposure (Fig. 2C and D). Therefore, non-activated HLA-DR^- cells had the same susceptibility to induction of apoptosis due to RMD exposure than activated, HLA-DR^+ cells.

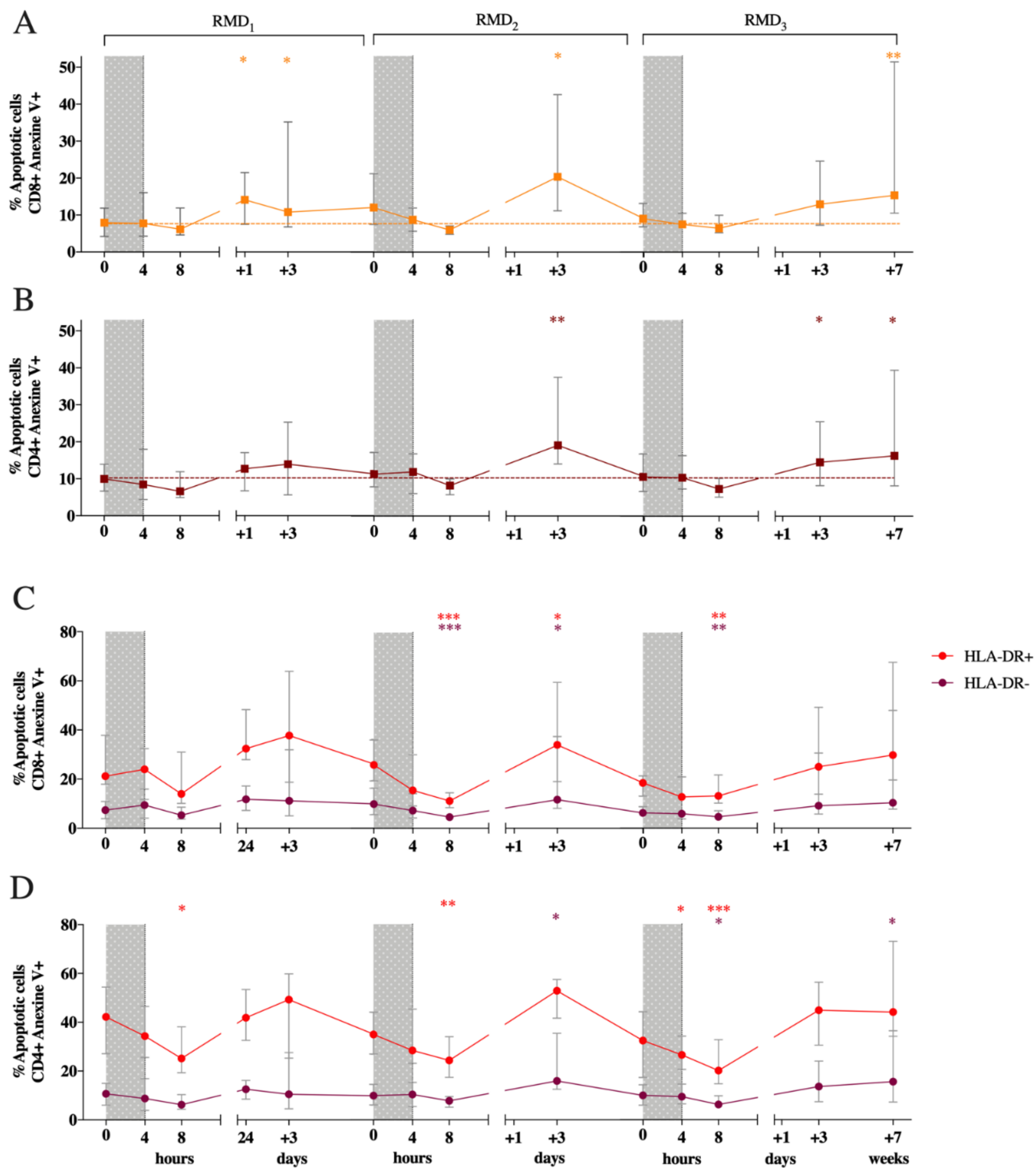


Figure 2: effect of romidepsin on viability on T-cells. Apoptotic cells (Annexin V$^+$/7AAD$^+$ and Annexin V$^+$/7AAD$^-$) percentages are shown for CD8$^+$ (A) and CD4$^+$ T-cells (B). Changes in percentages of apoptosis in cells expressing (in red) and non-expressing HLA-DR (in purple) are shown for CD8$^+$ (C) and CD4$^+$ (D) T-cells. Romidepsin administration doses are indicated by grey bars. Sampling time points at 4h, 8h, 3 days and 7 days after each administration are indicated. Median with interquartile range is shown. P-values (* p<0.05 ** p<0.01 ***p<0.001 **** p<0.0001) are indicative for the corresponding timepoint compared with respective preRMD$_1$.

CD8$^+$ and CD4$^+$ naïve populations increase after MVA$_1$ and up to 24 hours after 1st romidepsin exposure followed by a shift to memory phenotypes.

As activation status did not impact susceptibility to RMD induced apoptosis, we next assessed whether RMD could affect the distribution of different T differentiation subsets in the peripheral blood. Flow cytometry was used to measure the frequency of CD4$^+$ and CD8$^+$ differentiation subsets defined by CCR7 and CD45RA expression at each timepoint. Three weeks after MVA$_1$ the frequency of naïve CD8$^+$ T cells increased from a median frequency of 30.5% to 35.2% and up to 49.6% 24h after RMD$_1$ (Wilcoxon signed-rank p=0.0122 and p=0.0005 respectively, Fig. 3A). Similar results were observed for naïve CD4$^+$ T cells which increased from 33% at baseline to 35.2% 3 weeks after MVA$_1$ and up to 41.7% at 24h after RMD$_1$ (Wilcoxon signed-rank p=0.0227 and p=0.0110, respectively). Seven days after RMD$_1$, frequency of CD8$^+$ and CD4$^+$ naïve cells were not further increased after RMD$_2$ or RMD$_3$. On the contrary, frequency of both CD8$^+$ and CD4$^+$ naïve T cells decreased after RMD$_2$ and RMD$_3$ while the median frequency of CD8$^+$ effector memory and CD4$^+$ central memory cells progressively increased over RMD$_{1\text{-}2\text{-}3}$. T-cell differentiation subsets were not further changed 7 weeks after MVA$_2$.

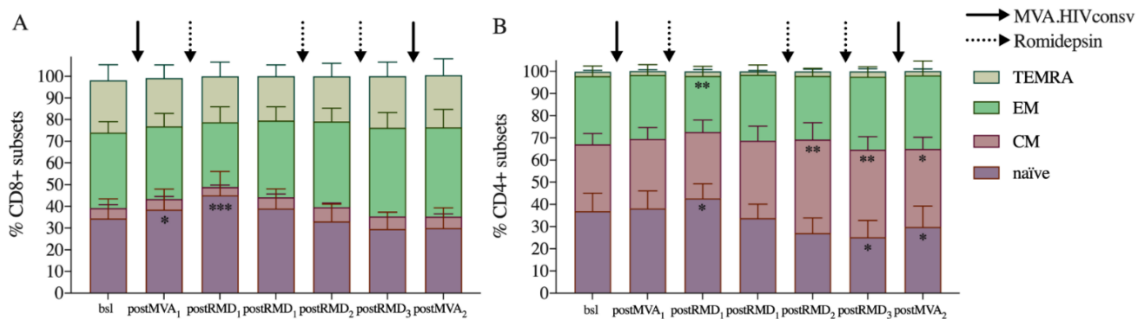


Figure 3: Characterization of T cell subsets over the course of the intervention. Average of relative frequency of CD8$^+$ (A) and CD4$^+$ (B) T cell subsets based on CCR7 and CD45RA expression is shown. TEMRA: highly differentiated effector cells expressing CD45RA (CCR7$^-$/CD45RA$^+$), EM: effector memory cells (CCR7$^-$/CD45RA$^-$), CM: central memory (CCR7$^+$/CD45RA$^-$) and naïve (CCR7$^+$/CD45RA$^+$). P-values (* p<0.05 ** p<0.01 ***p<0.001 **** p<0.0001) are indicative for the corresponding timepoint compared with respective bsl.

Romidepsin treatment increases T-cell activation, especially in vaccine-specific T cells

In order to assess the effect of RMD on T-cell activation and T cell exhaustion markers, we evaluated frequency of $CD4^+$ and $CD8^+$ T cells expressing HLA-DR, CD69, CD25 and PD-1. A peak frequency of $CD8^+HLA\text{-}DR^+$ T cells was observed 3 days after RMD_1 (Wilcoxon signed-rank, $p<0.0001$) and increased levels were maintained over the course of the 3 RMD doses, as their median frequency more than doubled (4.1% at baseline to 9.6 % one week after RMD_3, Wilcoxon signed-rank, $p=0.0002$; Fig. 4A). The same results were observed in $CD4^+$ T cells, with levels of HLA-DR expression increasing three days after RMD_1 (Wilcoxon signed-rank, $p<0.0001$) and with twice as many HLA-DR expressing cells at day 7 after RMD_3 (median 3.1% at baseline to 6.3%, Wilcoxon signed-rank, $p=0.0017$; Fig. 4A) compared to baseline. The largest increase in percentage of cells expressing HLA-DR in both $CD4^+$ and $CD8^+$ T cells was observed in effector memory (EM) and highly differentiated effector cells expressing CD45RA (TEMRA) (Supl Fig. 3).

Importantly, upon *in vitro* stimulation with peptides covering the immunogen sequence, changes in the $HLA\text{-}DR^+$ expression in both, HIVconsv-specific $CD8^+$ and $CD4^+$ T cells - were already detected after MVA_1, with increases being detectable earlier and up to higher levels compared ot the changes seen in total $CD8^+$ and $CD4^+$ T cells. Percentage of $HLA\text{-}DR^+$ in HIVconsv-specific $CD8^+$ cells increased from a median of 5.1 % at baseline to 10.11 % 3 weeks after MVA_1, and up to 21% 3 days after RMD_1 (Wilcoxon signed-rank $p=0.0245$ and $p=0.0002$, Fig. 4C) and percentage of $HLA\text{-}DR^+$ in HIVconsv-specific $CD4^+$ cells increased from a median of 4.2 % at baseline to 8.3% 3 weeks after MVA_1, and up to 21.8 % 3 days after RMD_1 (Wilcoxon signed-rank $p=0.5614$ and $p=0.0023$, Fig.4D). High levels of activation were maintained during all RMD doses (Fig 4B and C) and reached the peak 3 days after RMD_3 suggestive of an additive effect of RMD in the vaccine-induced activation.

As changes in histone deacetylation and induction of viral transcription appear to very fast upon RMD exposure [22], we assessed in addition to HLA-DR, the expression of the early activation marker CD69 and CD25 in $CD4^+$ and $CD8^+$ T cells. Increases in the expression of CD69 in $CD4^+$ T cells were observed at the end of each RMD dose, being more pronounced after RMD_2 and RMD_3. The largest increase in $CD4^+CD69^+$ frequencies was observed in the CM subset, which showed an increase from median baseline levels of 2.5% to 4.3%, 7.1% and 6.7% at the end of $RMD_{1\text{-}2\text{-}3}$ (Wilcoxon signed-rank, $p=0.1205$, $p=0.0107$, $p=0.0479$ respectively) and in the TEMRA subset that

displayed an increase from median baseline levels of 2.7% to 4.5%, 9% and 6.6% at the end of RMD_{1-2-3} (Wilcoxon signed-rank, p=0.0833, p=0.0203, p=0.0315, respectively). On the contrary, changes in CD69 expression on $CD8^+$ T cells were less pronounced over the course of the intervention (Supplementary Fig 4A). As with HLA-DR^+, expression of CD69 returned to baseline levels 4 weeks after last vaccination. No significative changes in expression of CD25 were observed in $CD8^+$ or $CD4^+$ T cells.

Interestingly, HLA-DR^+ or $CD69^+$ expression was not increased further upon MVA_2 in $CD8^+$ or $CD4^+$ T cells. Also, levels of both activation markers in total and HIVconsv-specific T cells were restored to baseline levels 4 weeks after MVA_2 which might translate an exhausted status of activated T cells. The expression of PD-1 increased rapidly in $CD8^+$ T cells from median baseline levels of 4.6% to 6.4%, 7,2% and 6.1% at the end of RMD_{1-2-3} (4 hours) (Wilcoxon singed-rank, p=0.0215, p=0.0238 and p=0.0574 respectively, Fig 4D). The same kinetics were observed in $CD4^+$ T cells which increased where the frequency of PD-1 expressing cells raised from median baseline levels of 8.5% to 11.9%, 12.35% and 10.7% at the end of RMD_{1-2-3} (4 hours) (Wilcoxon singed-rank, p=0.0004, p=0.0002 and p=0.0046, respectively). However, in contrast to the additive effect of RMD_{1-2-3} on T cell activation markers, changes in PD-1 expression in both $CD8^+$ and $CD4^+$ T cells were transient, and levels of PD-1 expression were restored to baseline levels 3 days after each RMD dose.

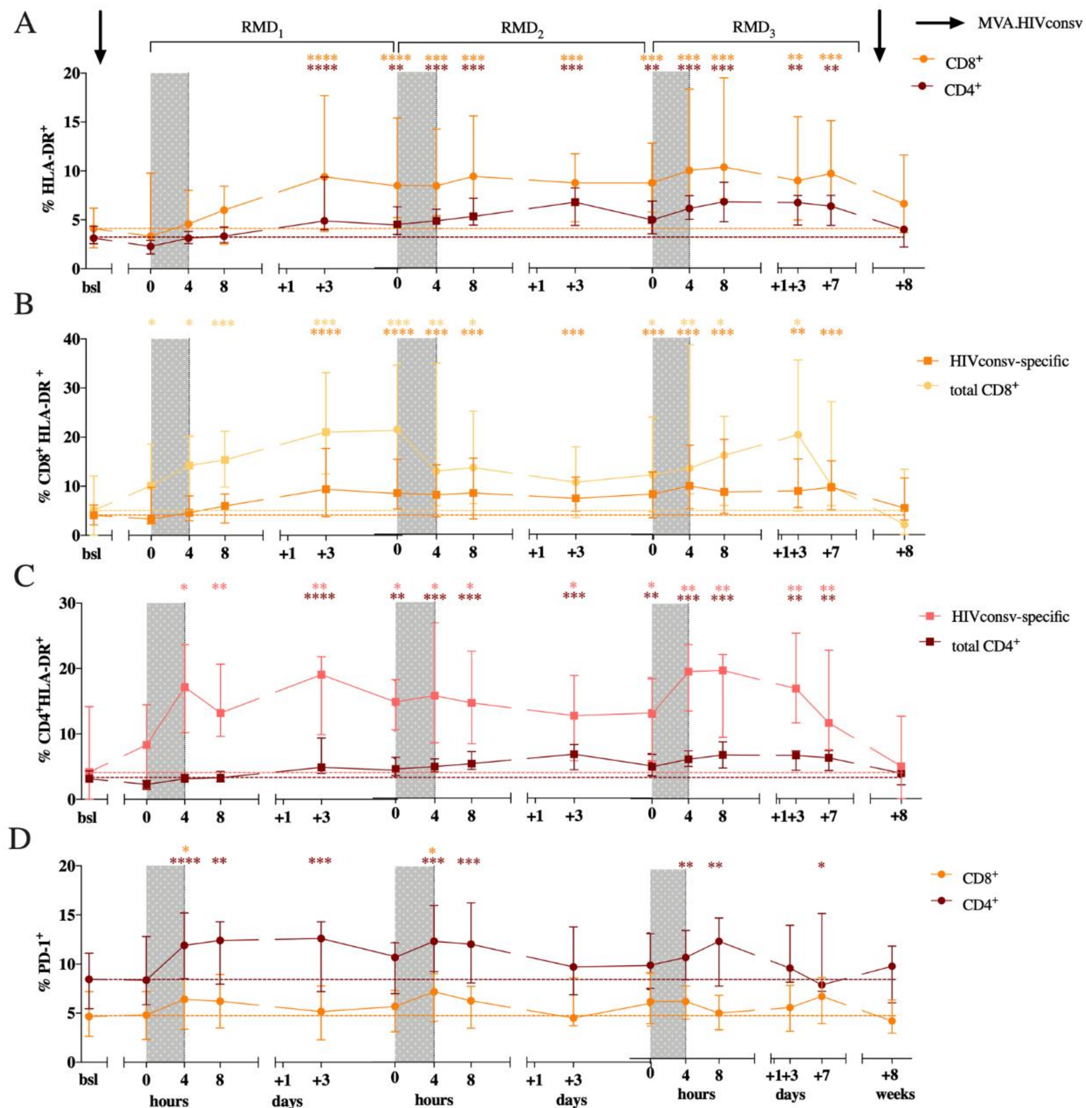


Figure 4: Characterization of T cell activation status over the course of the intervention. Percentage of HLA-DR expression is shown for $CD8^+$ in orange and $CD4^+$ T-cells in red (A). Levels of HLA-DR are shown for $CD8^+$ (B) and $CD4^+$ T-cells (C) in total and HIV.consv specific T-cells. Percentage of PD-1 is shown for $CD8^+$ in orange and $CD4^+$ T-cells in red (D). Median with interquartile range is shown. P-values (* p<0.05 ** p<0.01 ***p<0.001 **** p<0.0001) are indicative for the corresponding timepoint compared with baseline.

Polyfunctionality of HIVconsv-specific responses increased after MVA1 and decreased over romidepsin treatment

Considering the effect of RMD on cell death parameters, transient exhaustion and activation status, we assessed the effect of RMD on the functionality of vaccine-induced HIVconsv-specific T cells. Polyfunctionality of HIVconsv-specific T cells was measured by stimulation of PBMC with peptides covering the HIVconsv immunogen sequence and enumerating the cells producing IFNγ, IL-2, MIP1β and/or TNFα by flow cytometry. As

shown in Fig. 5, MVA_1 increased relative polyfunctionality in both $CD4^+$ and $CD8^+$ HIVconsv-specific T cells. Three weeks after MVA_1 ($postMVA_1$), the highest increase in polyfunctionality was observed in $CD4^+$ T cells that produced 2, 3 and 4 cytokines (post MVA_1, Figure 5), with a particular increase in INFγ secreting cells. On the other hand, $CD8^+$ T HIV-specific cells produced solely INFγ, IL-2 and TNFα and combinations mainly with 2 or 3 cytokines. However, during the treatment with RMD, the polyfunctionality was reduced to baseline levels in both HIVconsv-specific $CD4^+$ T and $CD8^+$ T cells and MVA_2 was not able to re-boost functionality profiles again (post MVA_2, Fig. 5). These results suggest that RMD treatment might have impaired functionality of vaccine-induced responses and prevented their normalization by subsequent boosting.

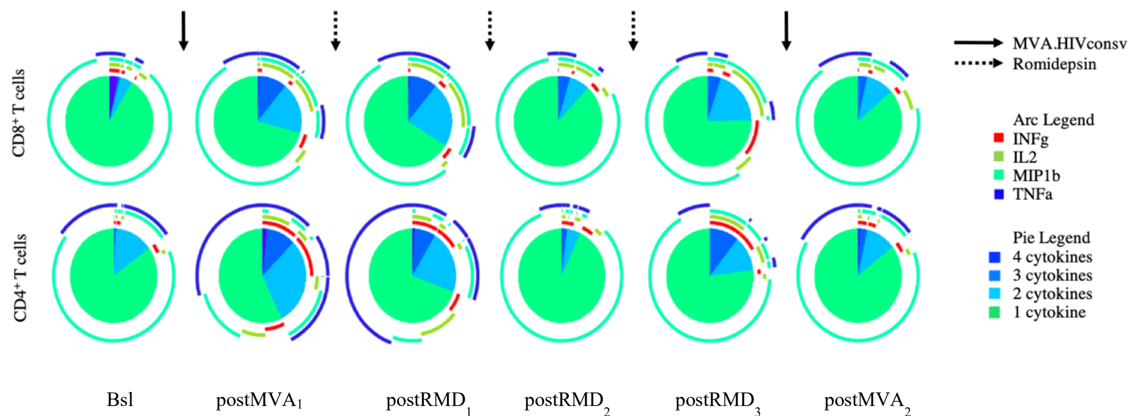


Figure 5: Characterization of T cell cytokines production over the course of the intervention. Polyfunctionality of $CD8^+$ and $CD4^+$ T-cells was analyzed by Boolean gating. Pie charts illustrate relative proportion of each of the different subsets (cells producing 1 cytokine, 2 cytokines, 3 cytokines or 4 cytokines).

Maintained antiviral activity of $CD8^+$ cells after romidepsin treatment

To further study the functionality of vaccine-induced T cells during the intervention in terms of effective killing capacity upon viral reactivation, we measured the *in vitro* antiviral capacity of $CD8^+$ T cells when cocultured with autologous, HIV infected $CD4^+$ T cells. The *in vitro* inhibitory capacity of PBMC-derived $CD8^+$ T cells was measured by standard viral inhibition assay (VIA) [12,27], infecting $CD4^+$ T cells with the two laboratory-adapted HIV-1 strains BaL (R5 tropic virus) and IIIB (X4 tropic virus). Inhibition of viral replication was measured by flow cytometry as the percentage of HIV Gag p24-positive CD4 cells. Inhibitory capacity was determined during the intervention at screening, 3 weeks after MVA_1 ($postMVA_1$), 1 week after RMD_3 ($postRMD_3$) and 4 weeks after MVA_2

($postMVA_2$). In contrast to the changes in cytokine production/polyfunctionality detected by flow cytometry, MVA_1 or $RMD_{1\text{-}2\text{-}3}$ did not alter the inhibition activity against HIV_{BaL} (median of 64%, 52% and 53,5% at screening, $postMVA_1$ and $postRMD_3$ time points at ratio 1:1) or HIV_{IIIB} viruses (median 46%, 44% and 43% at screening, $postMVA_1$ and $postRMD_3$ time points). In fact, there was a weak, but statistically significant increase in the inhibitory capacity after the second MVA vaccination given 4 weeks after RMD_3, at least at the 1:10 ratio for the HIV_{BaL} (median of 11% at baseline to 21% at $postMVA_2$, Wilcoxon signed-rank p=0.0200 for 1:10 ratio, Figure 6A) and for HIV_{IIIB}, (from median of 12% at baseline to 16% at $postMVA_2$, p=0.0156 for 1:10 ratio Figure 6B). The data indicate that antiviral activity, measured by VIA with laboratory-adapted viral strains was not negatively impacted during RMD treatment.

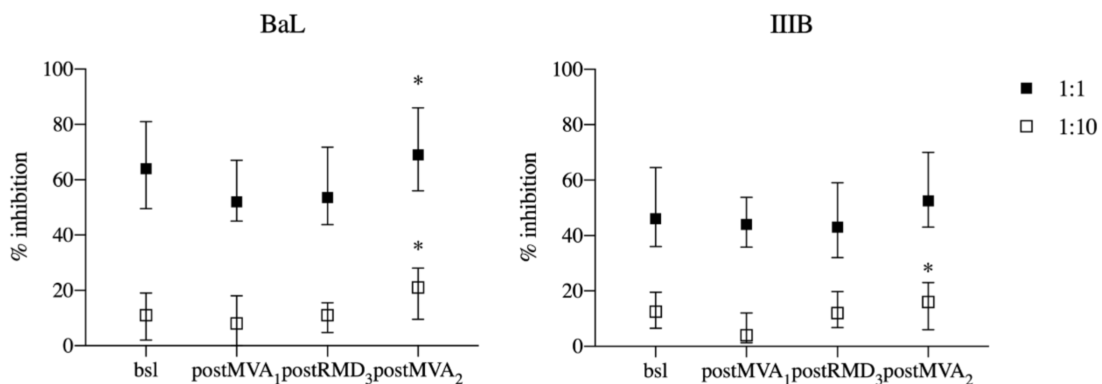


Figure 6: Viral inhibition capacity. Levels of CD8⁺ viral inhibitory capacity are shown for BaL (A) and IIIB (B) at two E/T ratios (effector/target ratio) 1:1 and E/T 1:10 in individuals that underwent the intervention (n=14 for BaL and n=15 for IIIB). P-values (* <0.05) are indicative for the corresponding timepoint compared with respective bsl.

Discussion

The BCN02 study was a kick&kill proof-of-concept trial combining MVA.HIVconsv vaccines with the HDACi romidepsin given as an LRA. The immune analyses in BCN02 showed a highly significant shift of HIV-specific T cell responses towards conserved regions and RMD resulted in marked increases in histone acetylation and induction of viral transcription. However, the ultimate reduction of the viral reservoir was overall minimal after the intervention (Mothe, Rosas, et al, submitted). Low baseline levels of viral reservoir in the early-treated participants included in the BCN02 study, limited reversal activity of RMD and/or a potential toxicity of RMD [28,29] on vaccine-induced T cells might also have precluded the capability to observe a more pronounced reduction

in the size of the viral reservoir. Here, we evaluated the effect that the HDACi romidepsin had on T cell viability, activation and functionality in a trial that combined a highly immunogenic vaccine with romidepsin given in a 3 weekly 5 mg/m^2 BSA regimen.

Our data document a toxic effect of RMD on T cells 3 days after each RMD exposure. Although it seemed to be a transient effect and viability of T cells was partially recovered 7 days after RMD_1 and $RMD_{2,}$ there was an additive increase in apoptosis over the full regimen with T cell viability not being fully restored by 7 days after RMD_3. Jones et. al and Zhao et. al detected increases in cell death upon long term (18h and 72h time) *in vitro* exposure to RMD, but no cytotoxic effect was observed when T cells were exposed to RMD for 4h only, which is more similar to the in vivo regimen [28,29]. However when testing differential short- and long- term effects of LRAs, Clutton et al observed that cells exposed for 3 hours to romidepsin *ex vivo* and then washed and cultivated in the absence of RMD for 3 days in order to mimic *in vivo* exposure, viability was not changed after 3 or 24 hours, but was significantly reduced at 48 and 72 hours [30]. These results are in line with our results showing a peak of apoptotic T cells 3 days after each RMD infusion and could explain the transient decreases in peripheral CD4 T cell counts observed in BCN02 (Mothe, Rosas et al, submitted). Moreover, the cumulative effect on viability observed after 3 romidepsin doses is in line with the delayed effects of romidepsin suggested by Clutton. These results should be taken into consideration when designing kick&kill clinical regimens that involve repeated doses of romidepsin because the toxic effects of the LRA could abolish the vaccine-induced gains in HIV-specific T cells.

In agreement with the REDUC trial, T cell maturation subset distributions did not change long-term except from an initial increase in both, $CD8^+$ and $CD4^+$ naïve T cells after MVA_1 and 24h of RMD_1 followed by a progressive increase of $CD4^+$ central memory T cells in subsequent $RMD_{2\text{-}3}$ [22,31].

As RMD may induce T cell activation as a result of viral reactivation, which could also derive in increases in exhaustion levels prior to the induction of apoptosis, we evaluated frequency of $CD4^+$ and $CD8^+$ T cells expressing HLA-DR, CD69, CD25 and PD-1.We detected an additive increase in $HLA\text{-}DR^+$ activation markers in both $CD8^+$ and $CD4^+$ T cells over RMD treatment, that was more marked in HIVconsv-specific T cells and already observed after the first MVA.HIVconsv vaccination. These changes could reflect an improved antiviral response, induced by the chosen vaccination regimen [32,33]. In contrast, changes in levels of CD69 and PD-1 were more rapid, peaking at the end of each RMD dose, and transient over the RMD treatment. In line with our observation on CD69 longitudinal expression, an increase in $CD69^+$ expression has been previously described

upon romidepsin and other HDACi such as panobinostat and vorinostat treatment *in vitro* [34,35] and also *in vivo* given alone [22] or in combination with a vaccine [31].

Although it has been previously described that RMD can cause an impairment of T-cell and NK function *in vitro* [26,28,36,37]*[276–279][275–278]*, *in vivo* data demonstrated that RMD did not alter the proportion of HIV-specific T cells or inhibit T cell cytokine production [22], at least in the peripheral blood. However, vaccine-induced responses in the REDUC trial were lower compared to the responses induced by MVA.HIVconsv vaccines in early-treated individuals (Mothe 2019, Mothe-Rosas submitted). In BCN02, RMD treatment decreased relative levels of polyfunctionality that had been enhanced during the first MVA.HIVconsv vaccination but did not lead to lower levels than at baseline. Of note, relative polyfunctionality of T cells was increased in CD4$^+$ T cells after the first MVA.HIVconsv vaccination. This observation is in line with previous reports showing that MVA-vectored vaccination can have a more profound effect on improving the polyfunctionality and T effector memory phenotype in CD4$^+$ T cells than in CD8$^+$ T cells [38]. Nevertheless, cells producing one cytokine did not decrease over RMD exposure, in line with INFγ results presented by (Mothe, Rosas et al, submitted).

Our data also indicate that despite fluctuations in activation, maturation phenotypes and polyfunctionality, romidepsin treatment did not impair the in vitro antiviral capacity of CD8$^+$ T cells. While some *in vitro* studies have shown a diminished inhibitory capacity when CD8$^+$ T cells were exposed to RMD but not to other HDACi [39,40], such an effect has not been observed *in vivo* in clinical trials using romidepsin. In the REDUC trial, the viral inhibition assay showed a trend towards increased inhibitory activity post-immunization that was lost after romidepsin exposure, but overall, antiviral capacity did not significantly change over time. Similarly, we did not see a reduction in the antiviral capacity of T cells over the course of $RMD_{1-2-3.}$

Our results highlight the lack of association between assays used to characterize CTL functionality, such as multiparametric flow cytometry and *in vitro* inhibition assays. In our study, polyfunctionality of CD8$^+$ T cells decreased while antiviral capacity was maintained during romidepsin treatment. In addition, the MVA_2 booster vaccination did not augment the proportion of cell secreting multiple cytokines while a slightly increased *in vitro* suppressive capacity was observed. These data are consistent with other studies that have shown a disconnection between cytokine secretion and antiviral capacity [41,42], suggesting that polyfunctional profile of HIV-specific CD8$^+$ T cells is not directly associated with viral suppression capacity [27,43][90,93][90,93]. Whether antiviral capacity measured by standard *in vitro* VIA assay using laboratory-adapted viral strains will translate into

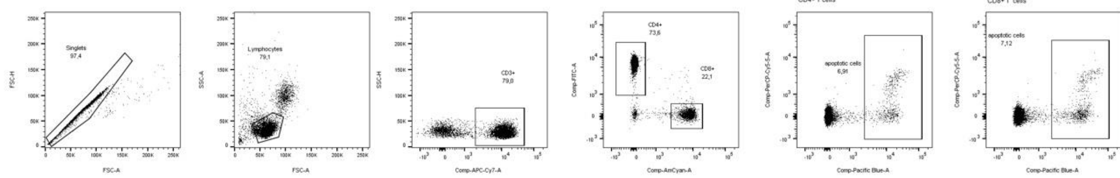
effective *in vivo* killing or reactivated, HIV infected cells remains to be determined. In addition, further work using autologous virus might be more representative of the physiological conditions in kick&kill strategies. Also, we acknowledge that dynamics of antigen presentation upon treatment with LRA, and whether these allow for an effective recognition and killing by $CD8^+$ T cells is still poorly understood [44].

This present study has a number of limitations which include the small sample size and the lack of control arms in BCN02, both placebo and single intervention arms to discern the effects exerted by romidepsin or the vaccine alone. Thus, the presented results need to be interpreted with caution. Still, the present study shows that romidepsin has a transient effect on T cell viability and exhaustion and increased cell activation in an additive way over 3 weekly doses. Although this can result in a decrease of polyfunctionality of vaccine-induced HIVconsv-specific responses, suppressive capacity of $CD8^+$ T cells was not impaired and should not preclude effective killing upon RMD-induced viral reactivation. These findings suggest that timing and order of LRA and T cell immunotherapy regimens are critical in order to achieve the clearance of reactivated latently HIV infected cells. Larger controlled clinical trials are needed to further investigate combinations of LRA and immune intervention in order to find the best strategy to achieve a functional cure of HIV.

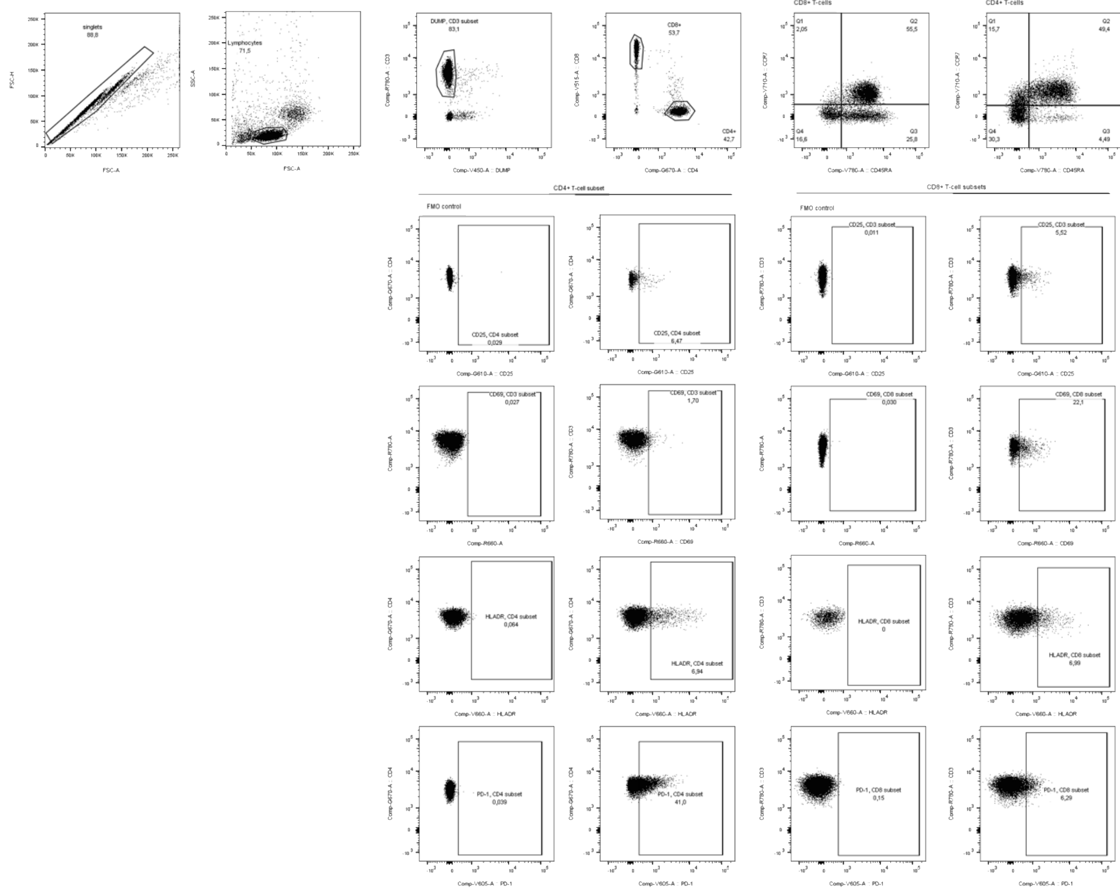
Acknowledgements

BCN02 trial was funded by ISCIII (PI15/01188) grant and Fundació Glòria Soler. These sub-analyses were partly funded by the European Union's Horizon 2020 research and innovation program under grant agreement 681137-EAVI2020 and by NIH grant P01-AI131568. Special thanks to all volunteers participating in this study for their perseverance and dedication, without whom the phase I clinical trial would not have been possible.

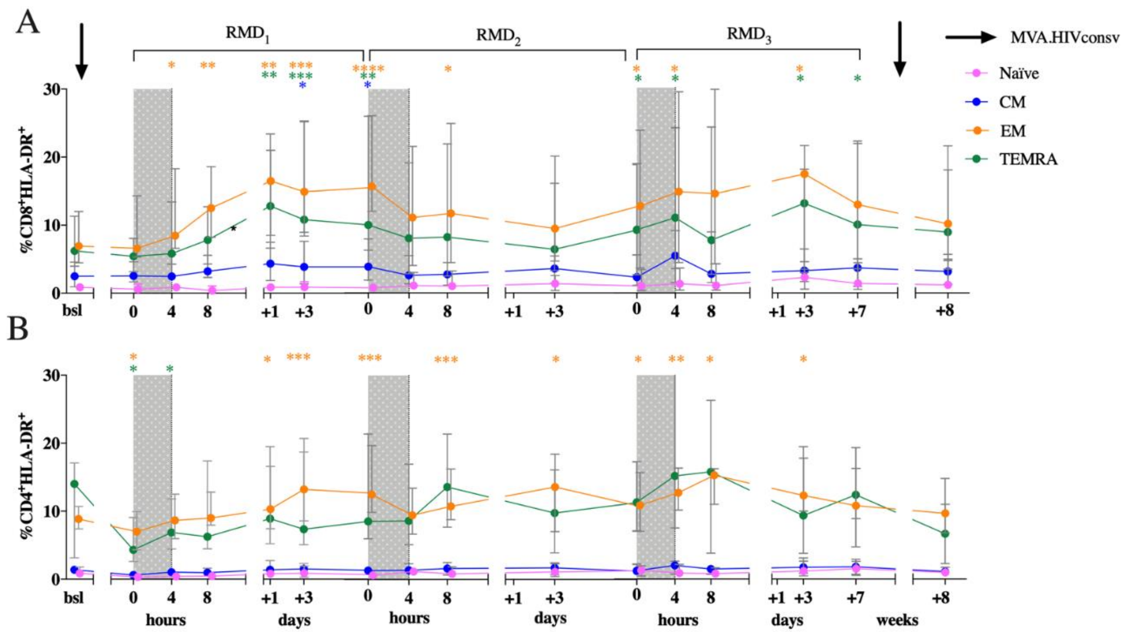
Supplementary material



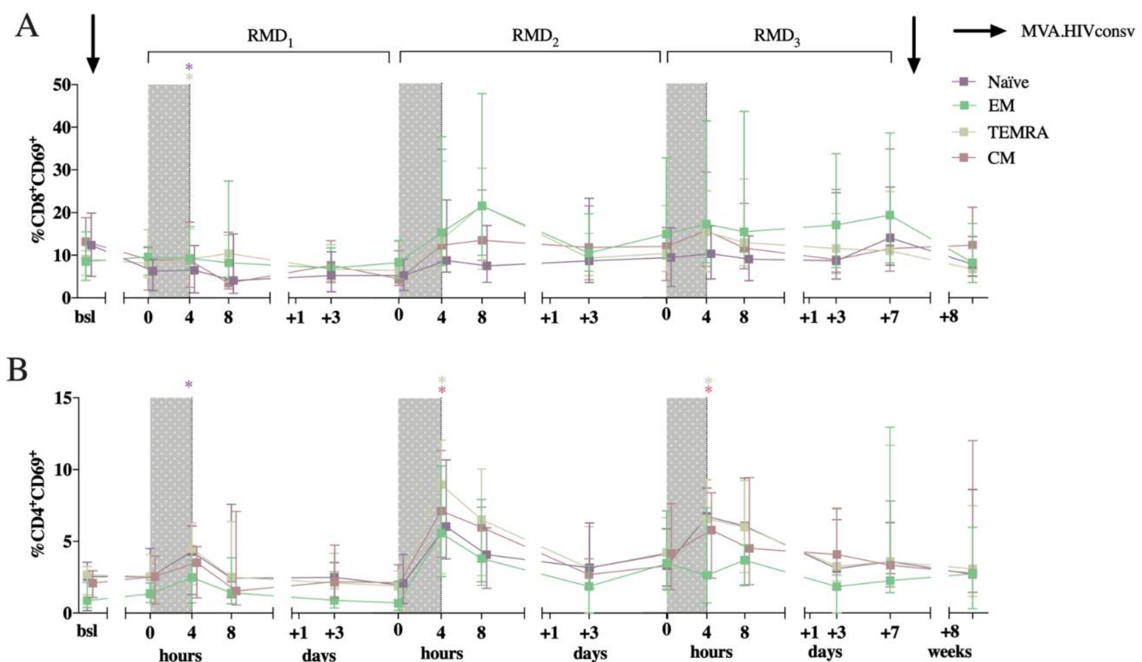
Supplementary Figure 1: gating strategy followed to determine percentage of apoptotic cells. A representative dot plot is shown. To identify apoptotic cells the gating strategy was the follow: A) Singlets, B) Lymphocytes, C) $CD3^+$ T cells, D) $CD4^+$ and $CD8^+$ T cells and E-F) apoptotic cells for $CD4^+$ and $CD8^+$ (Annexin V^+/$7AAD^+$ and Annexin V^+/$7AAD^-$).



Supplementary Figure 2: gating strategy followed to determine T-cell subpopulations, activation, exhaustion and cytokine production. A representative dot plot is shown. The gating strategy was the follow: Singlets, Lymphocytes , $CD3^+$ T cells, $CD4^+$ and $CD8^+$ T cells and CCR7 and CD45R was used to define memory cells. Within each subtype, CD69, CD25, HLA-DR, PD-1, IFNγ, TNFα, MIP1b and IL2 was measured and FMO was used as a control for each condition.



Supplementary Figure 3: Expression of HLA-DR in T cell subsets. Percentage of HLA-DR+ expressing CD8⁺ (A) and CD4⁺ T cells (B). Classification of T cell subsets is based on CD45RA CCR7 expression (naïve: CCR7⁺CD45RA⁺, EM:CCR7⁻CD45RA⁻, TEMRA: CCR7⁻CD45RA⁺ and CM CCR7⁺CD45RA⁻).



Supplementary Figure 4: Levels of CD69⁺ in T cell subsets. Proportion of CD8⁺ (A) and CD4⁺ T cells (B) subsets expressing CD69. Classification of T cell subsets is based on CD45RA CCR7 expression (naïve: CCR7⁺CD45RA⁺, EM: CCR7⁻CD45RA⁻, TEMRA: CCR7⁻CD45RA⁺ and CM CCR7⁺CD45RA⁻). P-values (* <0.05) are indicative for the corresponding timepoint compared with respective bsl.

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This declaration concerns the following article/manuscript:

Title: *In vivo* effects of romidepsin on T cell activation, apoptosis and function in the BCN02 Kick&kill clinical trial
Authors: Rosas-Umbert M, Ruiz-Riol M, Marszalek M, Fernández MA, Coll P, Manzardo C, Cedeño S, Miró JM, Clotet B, Hanke T, Moltó J, Mothe B and Brander C, for the BCN 02 study group.

The article/manuscript is: ☐ Published ☐ Accepted ☐ Submitted ■ In preparation

The phD student has contributed to the elements of this article/manuscript as follows:

A. No or little contribution
B. Has contributed (10-30%)
C. Has contributed considerably (40-60%)
D. Has done most of the work (70-90%)
E. Has essentially done all the work

Formulation/identification of the scientific problem	D
Planning the experiments and methodology design and development	D
Involvement in the experimental work/data collection	E
Interpretations of the results	D
Writing the first draft of the manuscript	E
Finalization of the manuscript and submission	D

The work presented in this Chapter was a project granted by FIS applied by Drs Mothe and Moltó. The phD student contributed to the grant application. The phD student performed relevant background literature search. The phD student was the primary responsible of designing all the experimental design and set up of new assays. The phD student performed all the experimental work (3 different flow cytometry panels and viral inhibition assays) and the data analysis.

Dr Christian Brander (director) Dr. Beatriz Mothe Pujadas (director)

DISCUSSION

The main objective of this thesis was to identify markers and mechanisms of immune control of HIV infection, in the setting of natural infection as well as in clinical trials using therapeutic T cell vaccines and latency reversing agents. The analyses were aimed to deepen our understanding of natural control of HIV and how these learning can be translated into effective therapeutic interventions that can lead to a functional cure and, with further improvements, may make viral eradication an attainable goal.

Need for an HIV cure

The development of the first antiretroviral therapy in 1996 has been one of the major successes in contemporary medicine, as it provided a measure to manage the spread of the HIV-1 pandemic that started in 1981 and prevented millions of premature deaths due to uncontrolled HIV infection [280]. In fact, current combination ART is highly effective suppressing HIV-1 replication, reducing the risk of transmission and preventing progression to AIDS. However, the disease remains uncurable due to viral reservoir that is established during the initial stages of the infection [281] and which contemporary antiretroviral treatment is not able to eliminate. Consequently, cART discontinuation results in a viral rebound within weeks in nearly all individuals. Moreover, cART is unfortunately still not available for all HIV-infected individuals, especially in resource limited countries, resulting in 38% of people living with HIV that have no access to antiretroviral treatment [11]. With close to 1.8 million new infections per year worldwide, there are still two urgent scientific challenges in the HIV field: the development of an effective prophylactic vaccine and attain a cure for those already infected.

In order to develop a cure for HIV, we need to understand the mechanisms by which the virus is able to persist upon cART. Several *in vitro* and animal models have been employed to further our understanding of how HIV establishes and maintains the latent reservoir [282]. Indeed, detailed knowledge of the molecular biology and cellular determinants of HIV latency is critical to identify new approaches and cellular and viral targets that could be employed to eliminate the latent reservoir. Complicating these approaches is that the elimination of the viral reservoir will probably require the reversal of viral latency and the simultaneous induction of an immune effector response able to eliminate the reactivated infected cells. Many drugs are being evaluated as potential latency reversing agents and both, T cell and B cell responses to natural HIV infection have been widely studied to inform vaccine immunogen designs. Several LRA and vaccine candidates have been or are currently been tested in clinical trials such as the ones discussed in chapters II-IV.

Interest in controller phenotype

An important goal of any HIV cure strategy that fails to achieve a sterilizing cure, would be to attain a functional cure of HIV-1 infected individuals, similar to the sustained suppression of viral replication seen in HIV elite controller (EC) individuals. Elite controllers, and long term non-progressors in general, are a small percentage of HIV-infected individuals (<2%) with natural capacity to spontaneous maintain undetectable or low HIV-1 viremia with normal CD4 T cells counts for long periods of time in the absence of cART. Even though these individuals may in part be biased for specific genetic markers, especially in the HLA region, they are of great interest as they represent a natural ability for functional cure of HIV infection [283,284]. Moreover, some studies have shown that EC have low HIV-1 DNA proviral levels [285,286]. Understanding the basis of this unique phenotype may help to define the key factors of HIV control that can be incorporated into novel immunogens and design of curative strategies. Some of the studies presented in this thesis further support the potential role of reservoir size in mediating HIV control and strategies to reduce reservoir size, prior to or during vaccination and treatment interruption may provide the necessary advances towards an effective functional cure.

Several factors have been described to play a role in HIV-1 control, including a number of host genetic, viral and immune factors [109,110]. As EC generally present a broad and strong $CD8^+$ T cell responses to HIV, it is believed that inducing this CTL activity would prompt the clearance of infected cells. However, latently infected cells do not actively present viral antigens and therefore are not susceptible to CTL mediated immune control and elimination. In addition, not all HIV targets induce CTL responses with the same functionality in terms of killing capacity. Indeed, specific HIV targets have been identified to be particularly enriched in $CD8^+$ T cell responses in individuals with low viral loads [234]. Of note, most of the studies performed in HIV controllers stem from cross-sectional analysis, and little is known on the evolution of the immune response over time in this group of individuals. In Chapter I, we aimed to longitudinally explore virological and immune factors involved in the loss of HIV control in individuals who previously had been able to naturally control their infection. We first showed that during loss of control -probably due to viral antigenemia- an expansion in the number of CTL responses occurred. However, this expansion was not observed for CTL responses targeting epitopes located in the identified beneficial regions and did not translate into significant changes in Gag-dominance patterns. We also showed that loss of control was preceded by a significant decrease in viral suppressive capacity by $CD8^+$ T cells and a

more exhausted CD8 T cell phenotype after loss of virus control. The data reinforce the idea that functional $CD8^+$ T cells are of great importance in the control of HIV replication in natural controllers. However, it remains unclear which factors may progressively impair CTL functionality in EC over time. In particular, while cross-sectional studies in large cohorts show the relevance of CTL escape in virus control, we were unable to demonstrate a significant increase in CTL escape mutations by viral sequencing HIV before and after loss of virus control. In addition, it is to be proven whether therapeutic strategies can reproduce an elite controller phenotype in individuals that do not present beneficial host genetic markers. Furthermore, understanding which additional elements of the immune system contribute to immune-mediated viral control in HIV-1 controllers is at this moment unclear but will be an essential information to more accurately evaluate the immunogenicity of therapeutic interventions in clinical trials and to inform on their effectiveness. Of note, future research in EC/LTNP is becoming more challenging in light of current treatment guidelines. With universal cART implementation and as new HIV infections are diagnosed and treated earlier regardless of HIV viremia, less individuals are being identified as HIV controllers [287,288].

A similar situation that may provide necessary insight into HIV control is the setting of post-treatment controllers, which has recently gained much interest.

Mechanisms behind the post-treatment control phenotype are not well understood and whether PTC are EC that could have controlled the virus even without a period of cART-supression, or whether they are two groups with two different phenotypes, and different control mechanism, still remains elusive [188,289]. In the first description of a group of PTC (the VISCONTI cohort), PTC were suggested to have different mechanisms of control compared to natural HIV controllers, especially in terms of HIV-specific $CD8^+$ T cell responses [290]. However, similarly to natural HIV controllers, PTC have very small viral reservoirs, both during cART and after treatment interruption [187]. Also, >50% of PTC are able to rapidly decrease plasma viremia after an initial peak as is observed in the CTL-driven viral decay phase in acute infection before viral setpoint is achieved [183]. In any case, as more trials in the HIV cure field are conducted in early-treated individuals and include a treatment interruption phase to assess efficacy of the intervention, more PTC will be identified that warrant further study. This possible new clinical phenotype has also provided new optimism regarding the possibility to achieve a functional HIV cure and it will be imperative to better understand the viral and immune mechanisms driving PTC so that these learnings can be harnessed for the development of future therapeutic interventions.

Assessing efficacy in HIV cure clinical studies

An important roadblock in designing effective vaccine candidates and developing promising immune cure strategies is the lack of reliable immune correlates of HIV-1 control. To date, no robust, functional immune biomarker has been identified that could serve as reliable predictors of viral control after a curative intervention [264–266]. This may of course partly be due to the lack of strong clinical effects of the interventions tested to date and highlights the need to develop more potent immune-based interventions in the future. In the absence of specific biomarkers or functional correlates of control, it still is necessary to ultimately validate any therapeutic/cure strategy by an analytical antiretroviral therapy interruptions (ATI) [265]. However, clinical trial designs that include an ATI phase have been very heterogeneous, in part driven by the concern that interrupting cART was associated with significant risks in some individuals. In the SMART study, it was demonstrated that episodic periods of cART guided by CD4 T cell counts between 250-350 cells/mm^3, was associated with a significantly increased risk of developing opportunistic disease or death from any cause, as compared with continuous antiretroviral therapy [268]. Based in this, short-term ATI a) are only recommended in clinical trials to test the efficacy of an intervention, b) are designed with restrictive inclusion/exclusion criteria and c) require a closed monitoring during the interruption until a pre-defined cART resumption criterion are met. Some ATI-trials in which the primary endpoint is the time to viral rebound, use frequent viral load monitoring and a low viremia threshold to trigger cART resumption. More recently, and especially in clinical trials in which an immune intervention is tested, a higher viremia may be allowed for a short period of time after an initial viral rebound to be able to measure an immune-mediated control [274]. Of note, even if they have largely failed in the past, treatment interruption studies have provided important information that can be used to iteratively improve future clinical trials. In this thesis, in the clinical trial RISVAC03 presented in the chapter II, the only factor associated with time to viral rebound after treatment interruption was the level of proviral DNA before interrupting cART, which is in line with other clinical trials [267–269]. Similarly, in the BCN02 trial we showed that having a low viremia before cART initiation was associated with controlling HIV-viremia for a longer period of time once cART was paused.

Furthermore, the design of new interventions including an analytical treatment interruption should take into consideration several factors such as reducing the risk of immune damage and reseeding of the viral reservoir. Higher nadir CD4$^+$ T cell counts as inclusion criteria for the ATI and absolute CD4 <350 cells/mm3 or CD4% decrease <15%

as cART restart criteria are accepted to mitigate the risk of further immune damage. As for the risk of reseeding of the viral reservoir, some studies suggest that after an expansion during the ATI, HIV DNA concentrations return to pre-ATI values within 6 months following ART reinitiation [291–293]. In Chapter II, we showed that levels of proviral reservoir did not increase after 2 weeks of treatment interruption although most of the participants had detectable levels of plasma viremia at the moment of ART reinitiation. This was further corroborated by the data in the BCN02 trial presented in chapter III, in which a conservative criterion of 2 consecutive plasma viral load determinations over 2,000 copies/ml was used as cART resumption criteria during the treatment interruption phase. In that study, ATI resulted in median time to resume ART of 28 days with median viremia of 19,250 copies/ml at the moment of ART resumption in 10 individuals. Of note, we did not observe viral reseeding either in the 3 individuals who were able to maintain low viremia up to 32 weeks, suggesting that there is not only a window of time but also a magnitude of viral replication to prevent re-seeding of the viral reservoir following treatment interruption.

As mentioned, lessons from clinical trials conducted in early-treated individuals have allowed to estimate PTC rates to be up to 13%. This observation has also strengthen the importance of considering longer periods of viremia before cART is resumed during the ATI and the inclusion of a placebo control arm in the clinical trial design to not misinterpret the intervention efficacy [220,225]. Nevertheless, the inclusion of placebo groups raises also several ethical questions (risk of transmission, reducing willingness to participate, participant exclusion from future trials, etc) and are only recommended when trials are well powered to perform statistical comparisons between groups and therefore, in early exploratory trials, historical controls for time to HIV rebound and estimated PTC rates could be considered [274].

Aside from measuring the viral reservoir and the immune response before the ATI, an additional indirect marker to investigate a partial effect of an immune intervention, is analyzing the sequences of the rebounding viral population during the interruption phase. Even if effective viral control is not achieved in individuals stopping treatment, the analysis of the rebounding viral population can provide valuable insights into the selective forces that virus-specific immune responses might exert once cART is interrupted. Such analysis have been highly informative in completed preventive trials, including STEP and RV144 phase III trials, which have shown an measurable impact of vaccination on breakthrough HIV-1 sequences due to the selective T-cell pressure exerted by the vaccine [294–296]. Therefore, a “sieve effect analysis” on the

breakthrough viral population is expected to be also informative in the therapeutic setting. In Chapter II, we analyzed the rebounding viruses in RISVAC03 clinical trial, but we did not detect a strong immune selection pressure on rebounding viral populations during the treatment interruption phase. We hypothesize that aside from a possible insufficiently strong vaccine-induced T cell response, RISVAC03 participants had initiated cART in chronic stages of HIV infection and therefore, already harbored highly adapted viruses, as observed in samples from pre cART time points in which large number of CTL escape mutations were present. Therefore, the detection of an impact of immune intervention on the rebounding viruses was probably limited.

Recent studies have expanded the characterization of the viral population to proviral DNA and cell-associated RNA in order to investigate the genetic composition of the latent reservoir. In a recent sub-study of the REDUC trial intervention, the sequencing of the reservoir and the rebounding viruses allowed to assess whether the vaccine resulted in a selection of the rebounding virus and could explain a decreased genetic diversity of cell-associated HIV-1 RNA and DNA following the intervention [297]. The study showed no evidence of selective immune pressure measured as sequence diversity in the proviral or rebounding viral population. Moreover, in line with our results, the data showed high levels of preexisting epitope mutations in the latent reservoir. In consonance with this results, it has been described that the vast majority (98%) of latent viruses already present CTL escape mutations unless ART is started early [177]. Therefore, the presence of CTL escape mutations in the latent viruses is an added challenge to viral eradication that needs to be solved by an appropriate boosting of the broad-spectrum viral-specific CTL response [177]. In the BCN02 trial, participants had initiated cART during acute/early HIV infection and thus, lower viral reservoir levels with less viral heterogeneity are expected. Sieve effect analyses that will compare pre-cART and rebounding viral sequences are currently ongoing and are expected to clarify whether or not any immune-pressure was exerted by HIVconsv vaccination in these early-treated individuals.

Another important consideration when assessing efficacy in HIV cure studies is how to measure the impact on the HIV reservoir. Several assays have been developed to measure HIV persistence, of which one is the measurement of cell associated DNA. However, PCR-based methods do not amplify the full-length provirus so they can overestimate the frequency of latently infected cells [298]. As an alternative, detection of cell-associated RNA is used as an indicative of viral transcription, especially in latency reversal experiments and in clinical trial that include a LRA, as it cannot be used as a

marker of latent reservoir. The quantitative viral outgrowth assay (QVOA) is the gold standard for measuring persistent replication-competent virus [299]. However, it requires a large number of cells which difficult testing other anatomical compartments with insufficient number of cells. Alternatively, single copy assay (SCA) allow for detection of viral loads with high sensitivity, below 20 copies/ml and up to 0.5 copies/ml of plasma [300], yet the replicative capacity of these viruses remains unclear. Given that large proportions of the integrated viral genomes may not encode for replication competent virus, more recently developed tests now also aim to capture not only the total copy number of integrated viruses but differentiate replication competent from non-replicating viruses [301]. In BCN02, as discussed in Chapter III, HIV DNA, HIV CA-RNA and SCA were used to measure the latency-reversing effect of romidepsin. Whilst we detected transient increases in cell-associated HIV-RNA levels during romidepsin treatment, the overall strategy did not translate into detectable viremia by SCA and the impact on the reservoir as measured by HIV DNA was minimal.

Lastly, one of the major caveats for the HIV persistence field is still the limited knowledge on the presence of HIV reservoir in other body compartments and cell types others than T cells and how to detect the viral persistence in those tissues as well as how to clinically handle effects of potential viral reactivation, such as in the central-nervous system. Therefore, more feasible, high throughput, sensitive and well-validated assays are needed to accurately measure the HIV-1 latent reservoir in the clinical setting.

As referred to earlier, Kick&Kill strategies are being investigated in the HIV cure field to combine molecules able to disrupt the viral latency by activating HIV transcription along with immune interventions that can elicit HIV-specific CTL responses able to clear infected and reactivated cells. There are many classes of latency reversing agents which act on different cellular pathways and which result in a variable increase in HIV transcription and/or virion production. A number of these compounds have been tested in clinical trials, including vorinostat, panobinostat, disulfiram, romidepsin, bryostatin, interleukin-7 and TLR9 agonists. These trials have shown that HIV latency can be disrupted *in vivo*, as an increase on transcription is detected. Yet, none of these trials has shown a reduction of the HIV reservoir [246–254] when the LRA was given alone. Although the regimens and the dose concentrations of the LRAs need to be further optimized, it is believed that further stimulation of the immune effector response will be needed in order to completely eliminate the reactivated infected cells. Several clinical trials are currently evaluating different combinational strategies using LRA together with immune modulators (https://clinicaltrials.gov).

To date, four clinical trials have tested a combination of LRA with a therapeutic vaccine, including the RISVAC03 (NCT01571466, [261]) and BCN02 (NCT02616874), both studies presented in this thesis. Two other kick&kill trials have been reported, the REDUC (NCT02092116) [262] in which Vacc-4x vaccine was combined with romidepsin in chronically treated individuals and the RIVER (NCT02336074) study, in which HIVconsv vaccines were combined with vorinostat in early-treated individuals, similar to the BCN02 study [302]. Among the LRA used in those trials, the use of romidepsin seem to be able to induce more robust changes in HIV transcription, compared to disulfiram and vorinostat tested in the RISVAC03 and RIVER trial, respectively. However, while in the REDUC trial, a 40% decrease in proviral DNA copy numbers was observed, in the BCN02 only a marginally significant reduction of 19% was observed. This differences could be explained by the fact that participants of BCN02 received cART treatment early in their infection, which is associated with a significantly lower proviral DNA [303]. Therefore, detecting a significant reduction in reservoir size after LRA treatment might be more challenging. Another hypothesis, supported by some *in vitro* studies [276,304], would be that romidepsin could have impaired functionality of vaccine-induced T cells in BCN02 study, and therefore preclude a more significant reduction of the viral reservoir. In fact, HIVconsv vaccination was highly immunogenic, able to induce a broad, strong and polyfunctional T cell response towards conserved regions of HIV in BCN02. Whether these vaccine-stimulated T cells were more susceptible to potential toxic effects during romidepsin treatment was addressed in the work presented in Chapter IV. Our results showed a transient increase in the frequency of apoptotic cells during romidepsin treatment and an enhanced activation of vaccine-induced T cells. We also observed that over romidepsin doses, the number of HIVconsv-specific T cells secreting multiple cytokines induced after vaccination was progressively reduced. In agreement with the REDUC trial, the increased apoptosis did not affect the T cell subset distributions long-term [250,305]. Also, both trials showed that despite fluctuations in activation, maturation and polyfunctionality phenotypes, antiviral capacity of $CD8^+$ T cells at the end of romidepsin treatment was not impaired [305]. If viral reactivation induced by romidepsin in BCN02 was enough to allow for an effective recognition and killing by vaccine-induced $CD8^+$ T cells or whether further romidepsin administrations could have induced a more pronounced effect on the viral reservoir remains to be determined.

As mentioned above, aside from viral reservoir and immune measurements, the efficacy of any potential kick&kill can only be assessed clinically by assessing viral rebound kinetics after a treatment interruption. Despite the lack of a control arm, 23% of

individuals showed durable viremic control up to 32 weeks after treatment interruption in the BCN02 trial, which is potentially higher than natural PTC rates [183]. Although the mechanism of post intervention control in BCN02 is not yet understood, all participants that maintained low levels viremia upon treatment interruption were among subjects with lower viral reservoirs and among the highest vaccine responders, which supports the feasibility of effective kick&kill strategies tested in some early-treated individuals with strong cellular immune responses to the virus. However, larger and placebo controlled clinical trials are warranted to further investigate new agents to reactivate the virus from latently infected cells combined with optimized, potent immune interventions in order to attain a clinically significant functional cure of HIV. Achieving these two components has proved to be one of the biggest scientific challenges, but continuous progress is being made in the field. The compiled work presented in this thesis aimed to provide insights on mechanisms of viral control, both in natural infection and post-intervention that can contribute into the design of future, effective HIV cure strategies.

CONCLUSIONS

Conclusions of Aim I. Identify virological and immunological parameters involved in abrupt transition to progressive HIV infection in a cohort of previous HIV controllers.

I. Loss of control (LoC) in previous HIV controllers was not related to a viral evolution towards a more immune-escaped virus although change of tropism was identified some individuals.

II. Loss of control was associated with an increased number of HIV-specific T cell responses after LoC, potentially due to antigen driven expansion. Loss of functionality of $CD8^+$ T cells, measured as a lower viral inhibition capacity and increased levels of CD38 and PD-1 expression, preceeded loss of viral control and could be used as predictors of a declining immune control of HIV.

Conclusions of Aim II. Integrate host and vaccine-induced virological and immunological parameters to define potential predictors of viral outcomes after a structured treatment interruption in a therapeutic vaccine trial.

I. Viral replication after treatment interruption was associated with increased T and B cell responses to HIV, possibly due to viral replication. Rebounding viremia precedes reservoir reseeding, as proviral reservoir did not increase after 2 weeks of treatment interruption even though half of participants had detectable plasma viremia.

II. MVA-B did no generate a strong immune selection pressure on rebounding viral population during treatment interruption.

III. Levels of HIV-1 proviral DNA was associated with time to viral rebound and peak viremia after treatment interruption. Reduced peak viremia was also associated with a decreased number of HLA class I allele associated polymorphisms in Gag sequences in the rebounding vials population.

Conclusions of Aim III. Evaluate the safety, immunogenicity and effect on the viral reservoir of the kick&kill strategy tested in the BCN02 clinical trial combining HIVconsv therapeutic vaccination with the LRA romidepsin in a cohort of early treated HIV^+ individuals.

I. The combined kick&kill intervention was safe, well tolerated and resulted in durable viremic control up to 32 weeks after cART in 23% of early-treated individuals.

II. Romidepsin treatment resulted in increases in histone acetylation, cell-associated HIV-1 RNA and in T cell activation, which was associated with a marginally significant reduction of the viral reservoir.

III. MVA.HIVconsv booster vaccinations increased breadth, magnitude and immunodominance of CTL responses towards the HIVconsv immunogen sequences.

IV. Plasma viral load before antiretroviral treatment initiation was the only factor significantly associated with the immune-driven control observed after cART interruption.

Conclusions of Aim IV. Evaluate the *in vivo* effects of the histone deacetylase inhibitor romidepsin on T cell populations and functionality of vaccine-induced T cells responses in BCN02 clinical trial participants.

I. Romidepsin had a transient, negative effect on T cell viability. Romidepsin increased activation marker expression of vaccine-induced T cells in a cumulative manner. T cell memory phenotypes did not change markly over romidepsin treatment.

II. Romidepsin decreased the levels of polyfunctional, multiple cytokine secreting cells induced by MVA.HIVconsv vaccination. Romidepsin did not reduce the suppressive capacity of $CD8^{+}$ T cells as measured in *in vitro* viral replication assays.

PUBLICATIONS

Published papers:

1. Beatriz Mothe, Nuria Climent, Montserrat Plana, Miriam Rosás, José Luis Jiménez, María Ángeles Muñoz-Fernández, María C. Puertas, Jorge Carrillo, Nuria Gonzalez, Agathe León, Judit Pich, Joan Albert Arnaiz, Jose M. Gatell, Bonaventura Clotet, Julià Blanco, José Alcamí, Javier Martinez-Picado, Carmen Alvarez-Fernández, Sonsoles Sánchez-Palomino, Alberto C. Guardo, José Peña, José M. Benito, Norma Rallón, Carmen E. Gómez, Beatriz Perdiguero, Juan García-Arriaza, Mariano Esteban, Juan Carlos López Bernaldo de Quirós, Christian Brander and Felipe García on behalf of the RISVAC-03 Study Group. *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.* The Journal of Antimicrobial Chemotherapy, June 2015

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Beatriz Mothe*, Miriam Rosas-Umbert*, Pep Coll, Christian Manzardo, Maria C. Puertas, Sara Morón-López, Anuska Llano, Cristina Miranda, Samandhy Cedeño, Miriam López, Yovaninna Alarcón Soto, Guadalupe Gómez Melis, Klaus Langohr, Ana M. Barriocanal, Jessica Toro, Irene Ruiz, Cristina Rovira, Antonio Carrillo, Michael Meulbroek, Alison Crook, Edmund G. Wee, Jose M. Miró, Bonaventura Clotet, Marta Valle, Javier Martinez-Picado, Tomáš Hanke, Christian Brander and Jose Moltó for the BCN02 study investigators. *HIVconsv vaccines and romidepsin in early-treated HIV-1-infected individuals: Safety, immunogenicity and effect on the viral reservoir (study BCN02).* Submitted to Science Translational Medicine

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