Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen

Departement Biomedische Wetenschappen

Predictive biomarkers of a functional cure for HIV

Predictieve biomerkers voor de functionele genezing van HIV

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Chapter 1 - Human Immunodeficiency Virus

The Human Immunodeficiency Virus (HIV) is the causative agent of the Acquired Immunodeficiency Syndrome (AIDS) [1, 2] and has infected more than 70 million people since the start of the epidemic [3]. Worldwide about 35 million people have died from HIV while an estimated 36.9 million [31.1–43.9 million] were living with HIV by the end of 2017. Globally, an estimated 0.8% [0.6-0.9%] of adults aged 15 to 49 years are living with HIV. Africa still carries the highest burden of the epidemic, with 25.6 million people (69%) or nearly 1 in every 25 adults living with HIV (Fig. 1).

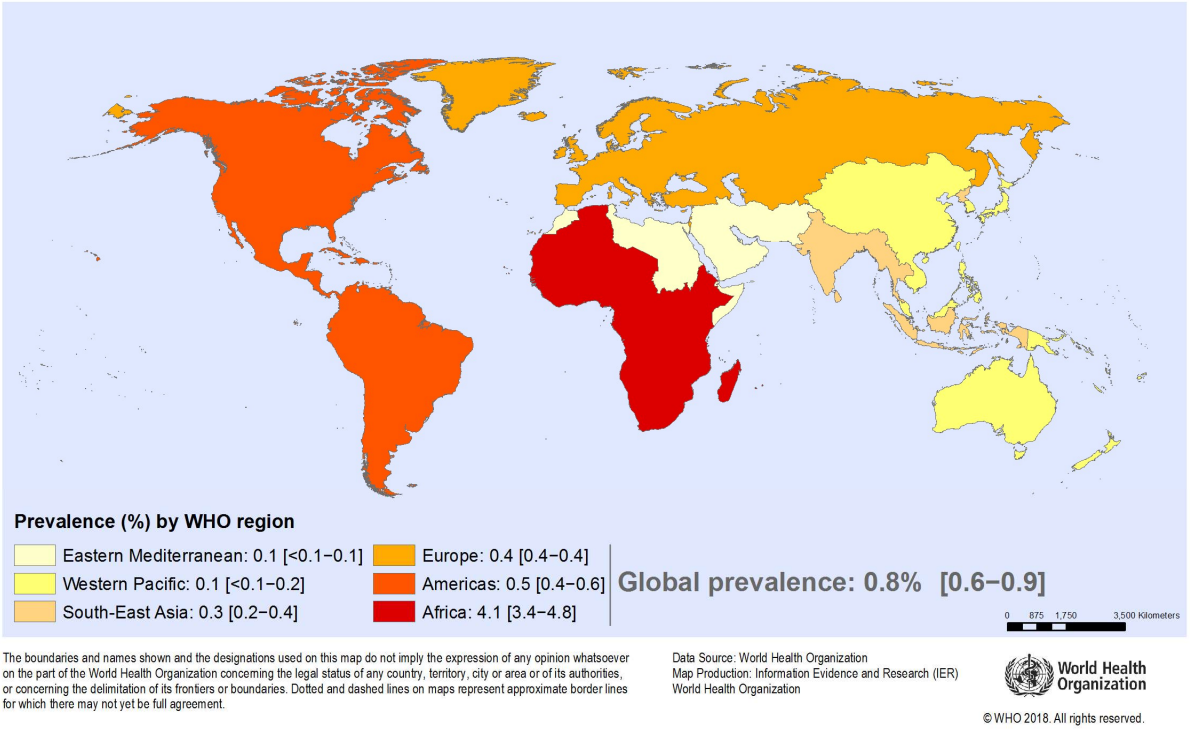

Figure 1. Estimated number of people living with HIV in 2017 by WHO region (figure adapted from www.who.int).

In 2017, 1.8 million people got newly infected with HIV and 940,000 HIV-related deaths were registered. In its current strategy to fight HIV, UNAIDS has set the ambitious 90-90-90 goal, meaning that 90% of infected people are diagnosed, 90% of known infected people are on treatment and 90% of people on treatment have suppressed viremia by 2020. With only little time left to reach this target, these parameters stood at 75-59-47 in 2017 globally (Fig. 2),

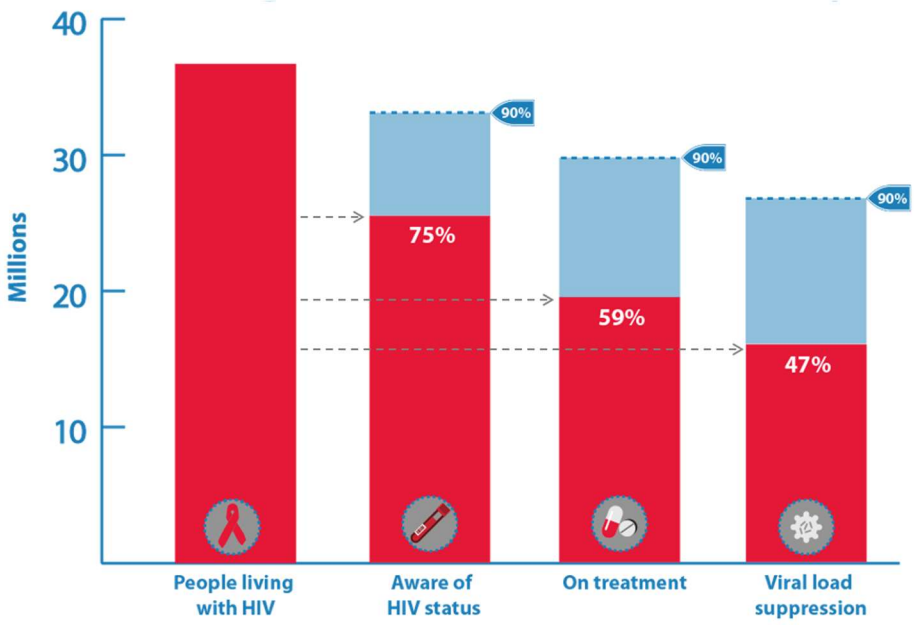

Figure 2. HIV testing and care continuum in 2017 worldwide (figure adapted from www.who.int).

indicating that the 90-90-90 goal will be very hard if not impossible to achieve by 2020 [4]. Belgium has reached this target, with indicators standing at 90.8-97.0-97.0 (data from 2017) [5].

With millions of new and undiagnosed infections every year, poor treatment coverage and rising levels of drug resistance, the HIV pandemic continues to become an ever more complex challenge for public health, especially in sub-Saharan Africa, making continued efforts in prevention and access to care absolute priorities in the fight against HIV/AIDS of today. Nevertheless, since it is questionable whether we will ever be able to "*treat ourselves out of this pandemic*" (Dr. KM De Cock), an actual cure for HIV is also urgently needed.

In this first chapter, a brief introduction will be given on the HIV virus itself, followed by an overview of different HIV cure strategies as well as descriptions of the HIV viral reservoir and the importance of CD8+ T cells in the control of viral replication.

HIV transmission

HIV can be transmitted through hetero -or homosexual contact, exposure to infected body fluids (blood transfusion, intravenous drug use) or from mother to child during pregnancy, delivery or breastfeeding [6, 7]. Factors increasing the risk of HIV transmission include the presence of sexually transmitted diseases, acute and late-stage HIV infection, and high viral load. Factors decreasing the risk of transmission include condom use, male circumcision, antiretroviral treatment, and pre-exposure prophylaxis [8].

HIV classification

HIV was first described in 1983 as a retrovirus belonging to the genus of lentiviruses [1, 2]. There are two types of HIV, named 1 and 2. HIV-1 is more virulent and the most common cause of AIDS worldwide. HIV-2 is less virulent and predominantly occurs in Western-Africa [9, 10]. HIV-1 is subdivided into four groups: M (major), O (outlier), N (new) and P. The major group M, which represents 90% of all infections, is again subdivided in nine subtypes: A-D, F-H, J-K but many recombinant forms occur as well. Several of these subtypes have distinct geographical distributions (Fig. 3).

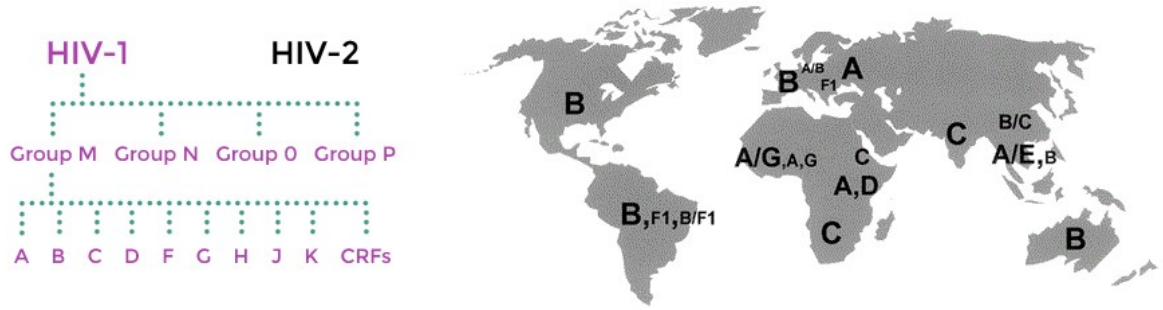

Figure 3. Classification of HIV (left, adapted from www.hiv.uw.edu) [*Common recombinant forms] and geographical distribution of HIV-1 group M subtypes (right [11])

Viral structure, genome and proteome

An HIV virion is spherical in shape and measures about 100nm. An outer coat, called the viral envelope, surrounds an inner capsid, which in turn contains the genome. The genome consists of two strands of positive sense RNA of about 9.2 kB long and is composed of nine genes encoding structural (*Gag*, *Pol* and *Env*), regulatory (*Tat* and *Rev*) and accessory (*Nef*, *Vif*, *Vpr* and *Vpu*) proteins [12] (Fig. 4).

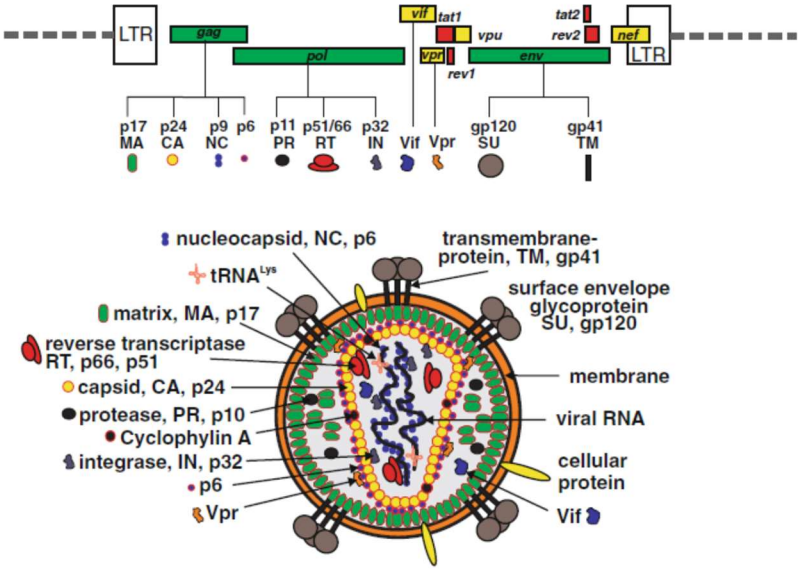

Figure 4. Structure of HIV virion (under) and HIV genome (above) adapted from Encyclopedia of AIDS DOI 10.1007/978-1-4614-9610-6_60-1.

The gag gene encodes the Gag (group-specific antigen) polyprotein p55, which is cleaved by the HIV protease into smaller proteins, including p24, p17 and p7, amongst others. The p24 capsid proteins surround the two RNA strands, which are tightly bound to the nucleocapsid (NC, p7). The matrix protein (MA, p17) is associated with the internal surface of the virion envelope [13, 14].

The pol gene encodes the Pol precursor protein p160, which is also cleaved by the HIV protease into the three viral enzymes required for replication (PR, p11; RT, p51/99; IN, p32).

PR is HIV protease, which processes the already mentioned long structural and enzymatic precursors into functional virus proteins. RT or reverse transcriptase-polymerase catalyzes the conversion of single-stranded RNA into double-stranded DNA. It has also RNAse activity to degrade the viral RNA after its reverse transcription into cDNA. IN or integrase is responsible for the integration of the pro-viral DNA into the host cell genome [15].

The env gene encodes the Env precursor glycoprotein (gp160), which is cleaved into two viral envelope glycoproteins (gp): gp120 and gp41 by a cellular protease furin. The viral envelope is composed of a lipid bilayer, derived from the host cell membrane, and contains on average 72 copies of spikes. Each spike consists of a "stem" of three gp41, anchored in the lipid layers, and is non-covalently associated to a "cap" made of three gp120 molecules. The gp120 subunit binds to receptors of the host cells such as CD4, chemokine receptors 4 and 5 (CXCR4 and CCR5) while gp41 is critical for catalyzing the fusion reaction between viral and host lipid bilayer membranes during virus entry [16].

The trans-activator of transcription (Tat) is a regulatory protein that plays an important role in early viral gene transcription. Tat is one of the first proteins produced after the start of viral transcription. Together with Rev, Tat is derived from early multiple-spliced HIV mRNA and strongly promotes transcription elongation by host RNA polymerase II [17, 18]. The regulator of viral protein expression (Rev) protein inhibits splicing of viral mRNA and promotes nuclear export of unspliced RNA and single spliced RNA species, which will be translated into structural enzymatic and accessory proteins [19, 20].

The Negative factor (Nef) accessory protein is responsible for the down regulation of host cell proteins such as the major histocompatibility complex I (MHC I) on infected cells. This

downregulation of MHC I dramatically decreases presentation of HIV peptides and thereby reduces the susceptibility to recognition and killing by cytotoxic T lymphocytes (CTLs) [21, 22].

The virion infectivity factor (Vif) accessory protein is essential for the assembly of competent virus particles and for the neutralization of host APOBECC3G, a protein inducing hypermutation during reverse transcription of the viral genome in infected cells [23]. Viral protein R (Vpr) is associated with the nucleocapsid and acts as a nuclear localization signal, thereby facilitating the nuclear import of the HIV genome [24]. Viral protein U (Vpu) downregulates the expression of CD4 and MHC I in order to evade immune recognition [25] and antagonizes the function of tetherin, an anti-viral host protein which inhibits the release of newly formed virions [26].

Viral life cycle

HIV primarily infects CD4+ T cells, macrophages and dendritic cells. To establish infection, HIV must first attach to its host cell by interaction of gp120 on the surface of the virus and the CD4 receptor and the coreceptors CCR5 and/or CXCR4 on the surface of the host cell. CCR5 is the physiological receptor for CC chemokines RANTES (CCL-5), MIP-1α (CCL-3) and MIP-1β (CCL-4) whereas CXCR4 binds CXC chemokines such as SDF-1 (CXCL-12). Most HIV clones use either CCR5 or CXCR4, but some can use both receptors. After attachment, the viral envelope and host cell membrane fuse by the action of gp41, resulting in entry of the nucleocapsid into the cell. Once the viral RNA is released into the cytoplasm of the host cell, RT makes a DNA copy of the viral RNA genome, simultaneously degrading the template RNA strand. Afterwards a complementary DNA strand is synthesized by RT and the ends of the resulting double stranded DNA are joined non-covalently, forming so-called LTR-circles. The resulting circular DNA is then moved into the nucleus and inserted into the host's cell chromosome by the viral IN

enzyme. The integrated viral DNA is from then on referred to as pro-viral DNA. Following integration, the pro-viral DNA can remain dormant (in resting latently infected cells) or after host cell activation, RNA may be synthesized from the pro-viral DNA, yielding messenger RNA (mRNA) and viral genomic RNA. Viral mRNA is transported to the cytoplasm and translated into enzymes and structural proteins. Some of the functional proteins are formed by cleavage of long polyproteins by the viral enzyme PR. Gp160 is cleaved by furin, a host cellular protease, to form gp120 and gp41 which are in turn inserted into the host cell membrane. Structural viral Gag-derived proteins surround the viral genome RNA to form the core. Finally, the virion is released by budding. After budding, autocatalytic processes ensure that PR is cleft from the Gag-Pol polyprotein to subsequently cleave the Gag and Gag-Pol polyproteins producing the matrix (p17), capsid (p24), nucleocapsid (p7), p6 proteins, RT, IN and PR itself. After these rearrangements, the virion is now mature and infectious (Fig. 5) [27].

Clinical course of infection

The course of infection can be divided into three stages: the acute stage, the chronic stage and the terminal or AIDS stage. In the acute stage, HIV infects mainly CD4+ T cells and produces massive amounts of virions, leading to a quick rise in viral load (VL) as measured in the blood plasma. Nevertheless, only a small proportion of infected patients develops symptoms, which are usually flu-like and include fever, fatigue, sore throat, skin rash, enlarged lymph nodes, diarrhea, nausea and general malaise. The sharp rise in plasma VL is accompanied by a quick decline in CD4+ T cell count. After about 6 weeks, HIV specific cellular and humoral immune responses peak and result in a rapid decline of VL and a (partial) restoration of the CD4+ T cell count. Plasma VL reaches a set-point level, initiating the chronic phase of the infection. During this chronic phase, VL remains relatively stable while the CD4+

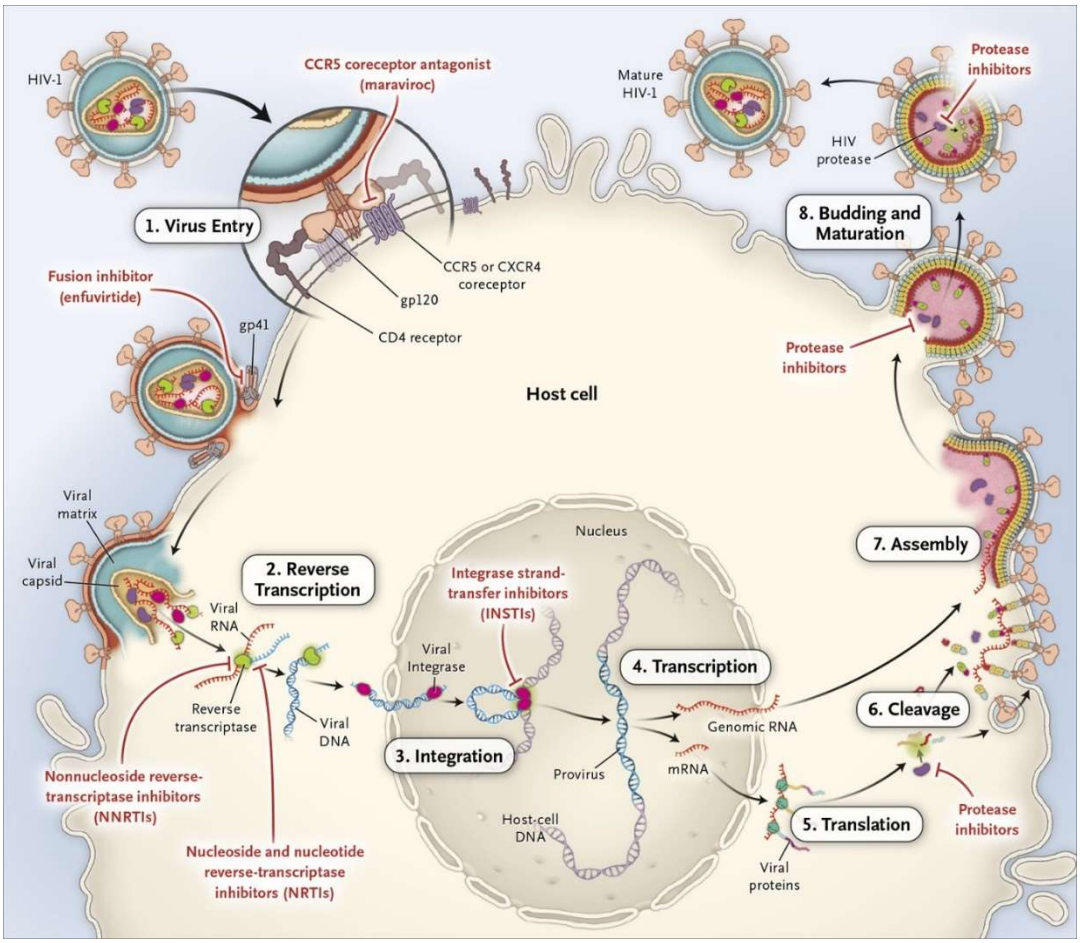

Figure 5. Life cycle of Human Immunodeficiency Virus Type 1 (HIV-1) and sites of action of the different classes of antiretroviral drugs [28].

T cell count slowly but steadily decreases. Clinical symptoms are limited however and usually do not raise suspicion of HIV infection. By the end of the chronic phase, which can vary from less than two years to more than ten years, the immune system becomes exhausted due to chronic immune activation and T cell depletion. In the terminal or AIDS stage, serious opportunistic diseases, including various infections and cancers, emerge and ultimately lead to AIDS-related death (Fig. 6).

A large majority (> 90%) of infected patients, called classic progressors, follows this slow course of infection and typically develops AIDS after about ten years. About 5% of patients however remain asymptomatic for more than ten years, have low to moderate plasma VL,

experience very little decline in CD4+ T cell count and are called long-term non-progressors (LTNP). An even smaller minority (1%) are so-called elite controllers (EC) and have VLs below 50 copies per mL for at least 1-2 years without therapy [29-31].

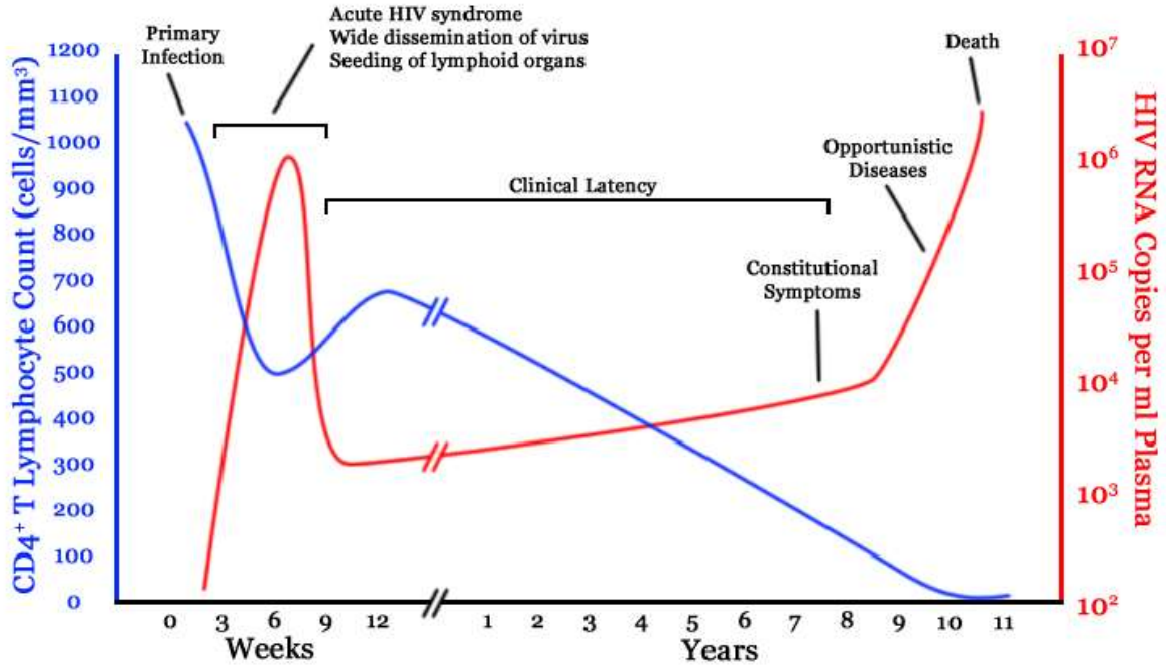

Figure 6. Clinical course of HIV infection (source: https://commons.wikimedia.org/wiki/File:Hiv-timecourse_copy.svg)

Anti-retroviral therapy

There are seven classes of anti-retroviral (ARV) drugs, each targeting a specific part of the HIV life cycle. These classes are summarized in fig. 5 and include nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs), fusion inhibitors (FIs), chemokine receptor antagonists (CCR5 antagonists) and entry inhibitors (CD4-directed post-attachment inhibitors). While mono -or bi-therapy quickly leads to development of drug resistance, combinations of three ARVs from at least two different classes effectively suppress viral

replication to below detectable levels. Nevertheless, ART is not curative, as it does not act on pro-viruses which are stably integrated in (mostly long-lived memory) CD4+ T cells. Most HIV positive patients therefore need to take ART life-long to prevent viral rebound [28, 32].

HIV latency and the viral reservoir

Within days after primary HIV infection, the viral reservoir is established, consisting mainly of long-lived resting memory CD4+ T cells containing a stably integrated pro-virus. In HIV infected individuals on cART, approximately 300 in 10^6 resting CD4+ T cells contain such a pro-virus [33]. Many pro-viruses however contain large internal deletions and/or hypermutations, and are unable to replicate or produce new infectious virions after reactivation. It is estimated that only about 2% of all proviruses are intact and replication competent (RC) [34, 35].

It follows that merely measuring the amount of HIV-1 DNA in CD4+ T cells does not give the most relevant reservoir size estimate, since a large majority is not RC. Fig. 7 gives a classification of all HIV-1 DNA present in infected individuals on cART and their respective clinical relevance for the viral reservoir.

- HIV-1 DNA can either be integrated as a pro-virus or occur in unintegrated linear or circular (episomal) forms. A small proportion of the integrated viral DNA can give rise to new infectious particles. Unintegrated DNA forms are considered to be products of failed integration events. It remains unclear whether they can be transcribed and give rise to viral protein expression. PCR-based assays can specifically distinguish each different form of integrated and unintegrated DNA [36].
- Full-length single-genome sequencing can determine whether pro-viruses are **intact** or whether they contain large internal deletions and/or hypermutations. While the latter are not RC, it has been shown that they can still be transcribed ('zombie' pro-

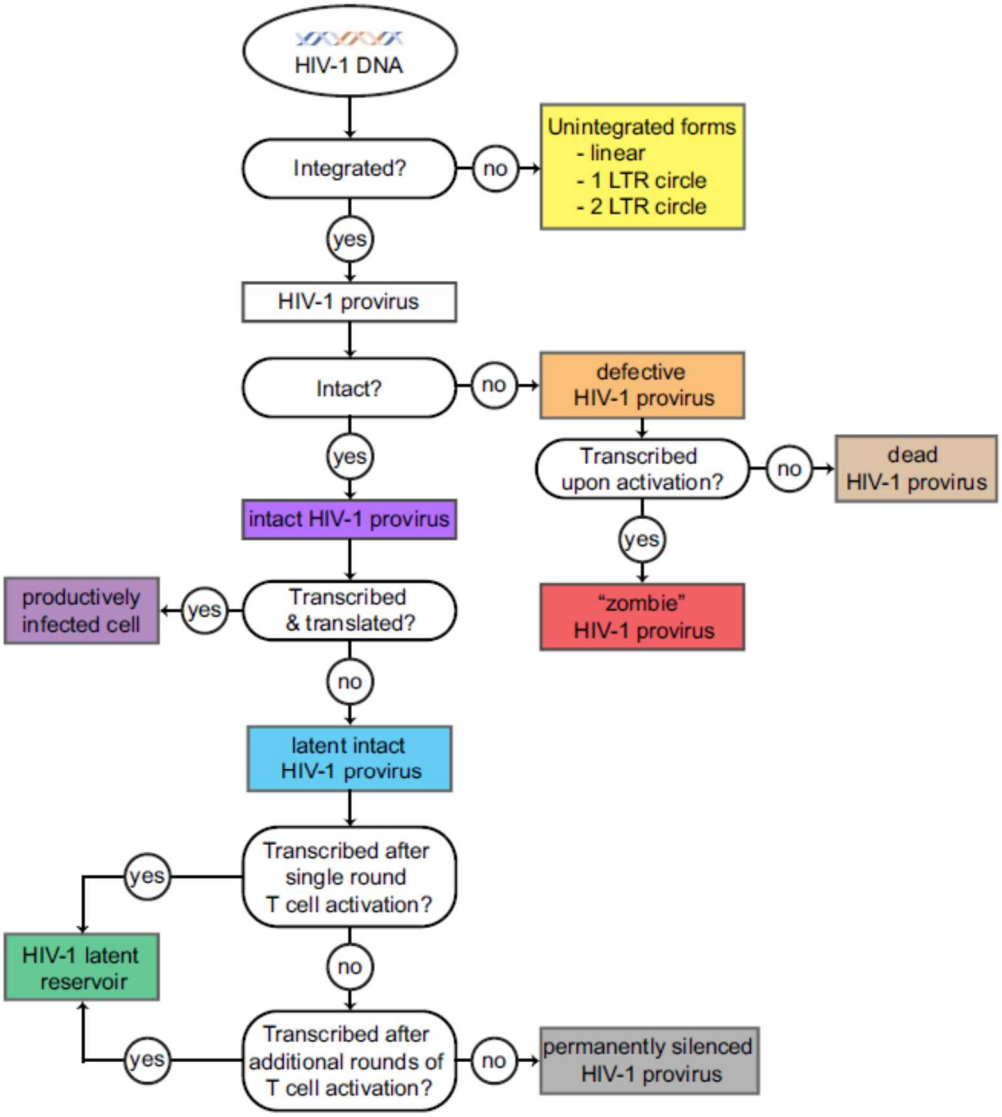

Figure 7. Classification of HIV-1 DNA present in HIV-1 infected individuals on cART (adapted from Wang et al. 2018 [37])

virus), lead to viral protein expression and therefore contribute to sustained immune activation/inflammation, which ultimately results in immune exhaustion and other complications, such as metabolic and cardiovascular disease. Defective pro-viruses which are not transcribed due to major genomic defects or irreversible epigenetic silencing are factually 'dead'. The primary target of cure strategies are intact pro-viruses, but zombie pro-viruses might be important to target as well in order to limit long-term negative effects of immune activation [38].

- When an intact pro-virus is actively being transcribed and translated, we speak of a productively infected cell. If not, we speak of **latent intact** pro-viruses.
- To determine whether a latent intact pro-virus can ever be reactivated and lead to new infections, quantitative viral outgrowth assays (qVOA) are used. These assays stimulate CD4+ T cells in-vitro and keep them in culture with feeder cells on which reactivated virus can replicate until a detectable concentration is reached. If a pro-virus is transcribed after one or several rounds of stimulation and produces new infectious virions, this pro-virus is considered to be part of the **HIV-1 latent reservoir**. Pro-viruses which cannot be reactivated anymore are considered to be permanently silenced and are not a target for HIV cure strategies [34, 39-41]. It is important, however, to consider that proviruses which fail to reactivate in-vitro still might be able to reactivate in-vivo.

Immune response against HIV

During the initial phase of HIV infection, after approximately ten days of localized viral replication at the site of infection (e.g. vaginal, penile or rectal mucosa), free virus or virus-bearing cells reach local draining lymph nodes, where the virus strongly replicates and starts spreading over the entire body. In the lymph nodes, dendritic cells (DCs) start presenting HIV peptides to naïve T and B cells, thereby initiating the adaptive immune response to HIV infection. In addition, DCs activate natural killer (NK) cells by producing interleukin-12 (IL-12), IL-15 and IL-18. DCs get activated by HIV and produce antiviral type I interferons. The function of DCs is however also impaired during infection, possibly due to HIV-induced IL-10 production by monocytes and regulatory T cells. This DC dysfunction might contribute to the lack of effective adaptive immune responses as well as proper NK cell activation [42-44].

T cell responses

About a month after infection, HIV-specific CD8+ T cells start suppressing viral replication, bringing down the initial peak of viremia via several mechanisms. Infected CD4+ T cells are killed through the direct cytolytic effects of perforin and granzymes. In addition, viral replication is also inhibited through the production of cytokines and other soluble factors. As a result of this CTL induced pressure on the virus however, targeted epitopes start to develop escape mutations [45].

Thus, while CD8+ T cells effectively suppress HIV replication in the first months following infection, escape mutations allow the virus to continue to replicate. A viral set point is reached when CTL induced suppression and escape through mutation have reached a balance. Lower viral set points are associated with a better clinical outcome and are likely explained by CTL responses targeting more conserved immunodominant epitopes, from which the virus can only escape at a high fitness cost. The human leucocyte antigen (HLA) type of a patient is an important factor determining which epitopes become immunodominant. Patients with protective HLA types (such as HLA-B27+ and HLA-B57+) target more conserved epitopes and as a result have better clinical outcomes, while in other cases (e.g. HLA-B35) the immunodominant epitopes are prone to continuous escape with poor control of viral load and rapid progression [46].

As the infection goes on, CD4+ T cells continue being killed on a large scale and as a result, T helper cell functions start waning. This strong immune dysfunction might explain the relative inefficiency of CD8+ T cells to respond to newly emerging escape mutants as compared to the initial, strong responses against transmitted founder viruses. Finally, CD8+ T cell responses also start to wane. Long-term antigen exposure and continued activation ultimately lead to T

cell exhaustion, with a loss in proliferative capacity and effector functions as a result. Immune checkpoint markers (negative regulatory molecules of immune activation) are upregulated on T cells during infection. These include programmed cell-death 1 (PD1), lymphocyte-activation gene 3 (LAG3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) [47]. Their appearance is considered as a sign of (chronic) activation and terminal differentiation. Interaction with their ligands on antigen-presenting cells can provide a negative signal to T cell function. The significance of these markers, however, is diverse and context-dependent. On the one hand, they are prominently expressed on fully functioning cell subsets (e.g. PD1 on follicular helper T cells and on cytolytic T cells). On the other hand, they also have a role in dampening the immune response against chronic antigenic stimulation, preventing hyper-activation and auto-immunity [48].

B cell responses

B cells are dysregulated quickly after infection due to high concentrations of virus and pro-inflammatory factors, leading to poor HIV-specific B cell and antibody responses. Changes in the intestinal mucosa during early HIV infection cause polyclonal B cell activation and germinal center destruction, with follicular lysis and B cell apoptosis as a result. Later in infection, germinal center B cells are increasingly activated and differentiated, leading to follicular hyperplasia in secondary lymph nodes. In late-stage HIV infection, increased deposition of collagen leads to fibrosis of secondary lymphoid tissues and ultimately to loss of homeostasis, reversal of immune-activating effects on B cells and finally a generalized loss of immune function [49, 50].

Pathways and barriers to HIV cure

About a decade ago, the 'Berlin patient' was the first person ever to be cured of HIV. After being diagnosed with acute myeloid leukemia and undergoing unsuccessful chemotherapy, in 2009 this patient received two rounds of total body irradiation (with complete destruction of his own immune system) and hematopoietic stem cell transplantation of C-C chemokine receptor type 5 (CCR5) Δ32 stem cells, which are resistant to infection by CCR5 tropic HIV [51]. The 'Berlin patient' has remained off cART ever since without any detectable viral rebound. Just this year, Gupta *et al*. reported on what might become the second person to be cured of HIV, called "the London patient". In the latter case, no irradiation was used and a single allogeneic hematopoietic stem cell transplantation (HSCT) with homozygous CCR5Δ32 donor cells was performed. The patient has been off treatment for more than 18 months with undetectable viral loads [52].

While these cases have generated a boost in HIV cure research, the followed therapeutic procedure is not feasible as a viable large-scale strategy for a cure. Nevertheless, several HIV-1 positive patients have been reported to achieve "spontaneous" viral control after stopping cART. This phenomenon is called post-treatment control (PTC) and, in contrast to a sterilizing cure, corresponds to a 'functional cure', i.e. a state of viral remission without the need for treatment [53-55]. As a result, understanding the factors which can explain the PTC status could be used to design strategies for 'functional cure'. In the last few years, our understanding of the interplay between the viral reservoir and the immune system has improved a lot and a number of barriers to achieve HIV cure have been identified.

As mentioned earlier, latency is established within days after infection [56] and consists of intact replication-competent pro-viruses which are not actively producing virus integrated in

the host genome. This latent viral reservoir is the main barrier to cure. While the majority of latently infected cells are long-lived resting memory CD4+ T cells, HIV persists in several other T-cell subsets and myeloid cells (mainly dendritic cells and macrophages) as well.

As already discussed, most infected cells contain proviruses with large internal deletions and hypermutations, and only a small minority is intact. As explained: while about 300 in 10^6 resting CD4+ T cells are infected in patients on cART [33], only about 2% contain intact proviruses [34, 35]. The gold-standard method to determine the proportion of cells that can be induced to produce replication-competent virus is the qVOA [40]. Based on qVOA, this proportion is about 1 in 10^6 CD4+ T cells. This discrepancy can in part be explained by reports that the classical qVOA does not induce all reactivatable proviruses [41]. However, it is currently uncertain whether all intact proviruses are in fact replication competent. As a result, there is still no consensus on how to measure the real size of the RC latent viral reservoir.

Another barrier to HIV cure is the fact that the viral reservoir can expand or maintain itself even in the absence of new infections, by proliferation of latently infected cells. It has been observed that up to 50% of the viral reservoir can consist of clonally expanded proviruses. Even though such clonal expansion has been reported in elite controllers as well and therefore does not seem to preclude a functional cure [57], it is important to identify the factors driving this clonal expansion, in order to design strategies to prevent it.

Most of the reservoir measurements are performed on cells in the peripheral blood, while probably 99 % of the infected cells are present in tissues throughout the body, which are difficult to sample in a representative way[58, 59].

Viral replication is effectively suppressed by cART, as evidenced by low levels of viremia in blood plasma, typically below the detection limit of routinely used assays (20 copies/ml

plasma). Still, poor penetration of drugs in tissues could allow residual replication of HIV-1 despite treatment adherence. Whether residual viral replication occurs in patients on cART and whether it plays a significant role in shaping the viral reservoir, is a long standing debate. The lack of viral sequence evolution and the absence of resistance development are the main arguments against residual viral replication [60, 61]. Evidence in favor is mainly based on cART intensification studies observing an increase in circular viral DNA, an indication of failed integration events [62].

Finally, certain tissue sites such as B-cell follicles in lymph nodes contain much more infected cells than blood [58, 59]. These sites are however more difficult to penetrate by cytotoxic CD8+ T cells, making immune interventions targeting latently infected cells more difficult [63, 64].

HIV cure strategies

Three different categories of cure strategies can be distinguished, namely (1) those interfering with the HIV life cycle, (2) those enhancing HIV specific immunity and (3) those modulating the immune system in general.

(1) A drastic way to **interfere with the HIV life cycle** is the transplantation of hematopoietic stem cells homozygous for CCR5Δ32 deletion. This has been tested on a small number of patients and has shown significant reductions in the size of the viral reservoir and delayed viral rebound after treatment interruption [52, 53, 65-67]. The mortality rate of the preliminary destruction of the patient's own immune system, prior to HSCTs is too high however to justify such an intervention without a clear clinical need. Another strategy to prevent new infections utilizes gene editing technology to modify CCR5 of the host. The concept is likely to be prone to resistance development however and so far no delayed viral rebound has been achieved with this

strategy in experimental models [68]. Another much studied strategy is the use of latency reversal agents (LRAs) to re-activate latent proviruses and thereby induce virus or immune mediated cell death ("shock and kill"). While clinical trials with LRAs have shown very moderate increases in viral expression, no reductions in reservoir size have been achieved [69-71]. More powerful, less toxic and more specific LRAs are needed. Finally, the "block and lock" strategy aims at completely silencing the latent reservoir, instead of reactivating and killing it. The use of *tat* inhibitors or RNA interference technology are being investigated and have already shown delayed viral rebound after treatment interruption [72].

(2) Two important strategies exist to **enhance HIV specific immunity**. One is the use of broadly neutralizing antibodies (bNAbs) which are able to neutralize free virus, eliminate viral antigen-expressing cells and enhance CD8+ T cell function [73-75]. Results from clinical trials are promising, with significant delays in viral rebound which are essentially determined by the pharmacokinetics of the used bNAb and the emergence of resistance [76, 77]. Current bNAb research is focusing on increasing half-life and using combinations of two or more antibodies so as to prevent resistance development. Alternatively, antibody based molecules with multiple specificities are being developed as well [78-80].

Therapeutic vaccination is the other important strategy to enhance HIV specific immunity by boosting host immune responses, more particularly CD8 T cells, in order to enable them to deplete virus-infected cells. Some studies combining this strategy with the use of LRA have already shown a moderate and temporary reduction in the size of the reservoir [81]. Such vaccines should probably target immune conserved sub-dominant epitopes to increase its chances of success, since it has been shown that the

viral reservoir contains many escape mutations in immunodominant epitopes [82, 83]. Immune CD8+ T cells of non-controller HIV infected patients on cART are however long term activated and exhausted, which may compromise the success of therapeutic vaccination. This strategy may need to be combined with an intervention which reverses this exhaustion, as discussed underneath.

(3) As mentioned above, the immune system of chronically infected HIV positive patients gets exhausted, as evidenced by the expression of a number of immune checkpoint (IC) markers such as PD1, LAG3, TIM-3, CTLA4, amongst others. These markers are **immune-modulating proteins** involved in negative feedback mechanisms that limit the activity of T cells after continued stimulation [84]. Such IC markers are increased on HIV specific T cells of patients on and off cART [85-87]. *Ex vivo* data has shown that treatment of these cells with antibodies blocking these pathways can restore their functionality [88]. Such antibodies are therefore potential candidates to boost general immunity. Several clinical trials are on-going to test the efficacy and safety of treatment with a single antibody or combinations of antibodies targeting several IC markers.

Chapter 2 – CD8+ T cell suppressive capacity of viral replication: review

Viral Inhibitory Activity of CD8+ T Cells in Human Immunodeficiency Virus Infection: review

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Introduction

A wide range of evidence supports the idea that CD8+ T cells play an important role in controlling viremia during human and simian immunodeficiency virus (HIV and SIV) infection. First, the emergence of HIV-specific CD8+ T cells during acute infection is quickly followed by a decline in viremia [89-94]. Second, the depletion of CD8+ T cells from SIV-infected rhesus macaques results in loss of viral control [95-97]. Finally, the emergence of mutations enabling viruses to escape CD8+ T cell responses indicates that a selective pressure is exerted by the same [98, 99]. Taken together, these observations clearly demonstrate the importance of CD8+ T cells in the control of HIV infection. Whether CD8+ T cells exert this control through mainly cytolytic or non-cytotoxic activities remains a matter of debate (reviewed in detail by McBrien *et al.* [100]) but is beyond the scope of this review.

In the search for an HIV-1 vaccine able to induce protective CD8+ T cell responses, robust assays are crucial to evaluate vaccine candidates. In this regard, the failure of IFN-γ enzyme-linked immunospot (ELISpot) and intracellular cytokine staining (ICS) to predict the failure of Merck's STEP trial showed the limits of these classical immunological assays [101, 102]. These assays are conceptually limited in that they only measure the production of one or several cytokines in response to a peptide stimulation, often at (high) peptide concentrations which might not even be biologically relevant. Earlier work often used the chromium release assay (CRA) which shows direct cytolytic activity of CD8+ T cells against HIV-infected CD4+ T cells [103]. However, besides being laborious, technically demanding and variable, the assay also gives little information about the CD8+ T cells themselves, other than that they can kill [104]. Alternatively, peptide-major histocompatibility complex (MHC) staining (e.g. tetramer) is used to determine the number of HIV-specific cells but gives little functional information about

these cells and can only be used with the limited number of known human leucocyte antigen (HLA) allele/peptide combinations [105]. Measuring proliferation in response to HIV peptides provides useful information but does not directly inform about the cytotoxic potential of proliferating CD8+ T cells. Taken together, it is probably more relevant to use assays which directly measure the actual inhibition of viral replication instead of using separate, indirect measures of CD8+ T cell functionality.

Such assays measuring the capacity to inhibit viral replication (named viral inhibitory activity or VIA) generally consist of co-cultures of superinfected CD4+ target T cells with CD8+ effector T cells and have been used since more than thirty years in HIV research [106]. Conceptually, this assay has the advantages of measuring inhibition of viral replication through both contact-dependent as well as independent mechanisms, and to measure exactly what an HIV vaccine aims to induce, i.e. suppression of viral replication.

In this review, we use studies dating from 1986 to 2018. We first summarize and discuss the different methods that have been described to measure *ex vivo* VIA. Next, we discuss how elite controllers (ECs) distinguish themselves from chronic progressors (CPs) in terms of VIA and discuss what other clinical parameters correlate with VIA. We give an overview of studies where VIA has been shown to be induced both *in vivo* as well as *in vitro* and finally, we discuss observed correlations with virological and immunological parameters.

Measuring VIA

As detailed in a number of papers [107-111], the main steps of a typical VIA assay are:

- Activation of target CD4+ T cells
- Preparation of effector CD8+ T cells:
    - directly *ex vivo* (resting) or

- after *in vitro* stimulation (expanded)

- Superinfection of target CD4+ T cells
- Co-culture of target and effector cells
- Measurement of viral replication

Underneath, we discuss these different steps in detail. Fig. 1 gives a flow chart of a typical VIA assay.

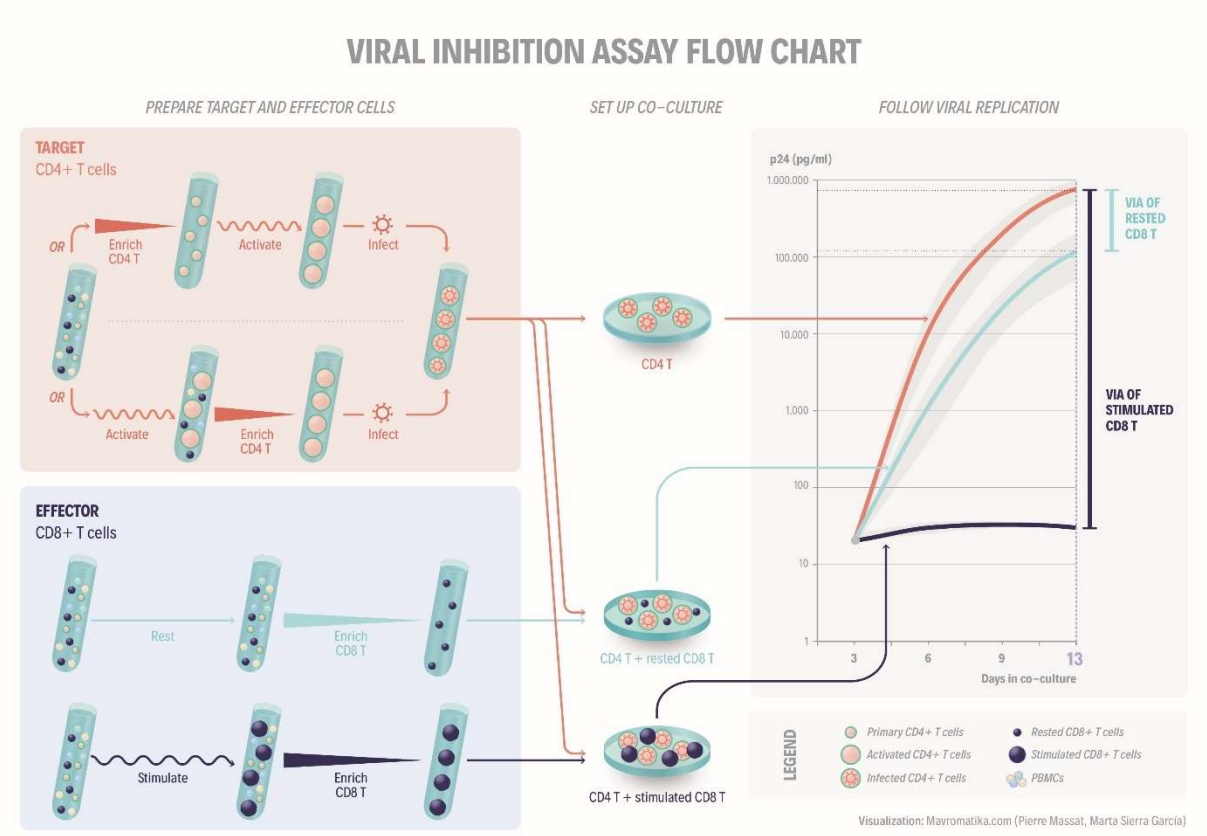

Figure 1: Flow chart of a typical viral inhibition assay. Target CD4+ T cells are enriched, activated and infected while CD8+ T cells are either rested or stimulated. Target cells alone and co-cultures of target and effector cells are set up after which viral production is monitored over time by measuring p24 concentrations in the supernatant. The difference in viral production is a measure of the viral inhibitory activity of the effector cells. This figure was made in collaboration with Mavromatika.com.

Target CD4+ T cell activation

Autologous CD4+ T cells are used as target cells when investigating contact dependent inhibition. Alternatively, non-contact dependent inhibition of viral replication can be studied using autologous CD4+ T cells in trans-well experiments or heterologous CD4+ T cells in co-culture [108, 112]. Other cell types, such as macrophages, can be used as target cells as well [113].

CD4+ target T cells are activated to render them more susceptible to infection. The most common agents used for stimulation include phytohemagglutinin (PHA) in combination with interleukin 2 (IL-2), beads coupled to anti-CD3 and anti-CD28 antibodies or bispecific monoclonal antibodies targeting CD3 and CD8. Depending on the agent and its concentration, target cell stimulation takes three to seven days. When using PHA + IL-2 or anti-CD3/CD28 beads, CD4+ T cells are enriched before stimulation. With bispecific monoclonal anti-CD3/CD8 antibodies, CD4+ T cells are enriched after stimulation, as the bispecific antibodies need to crosslink CD4+ and CD8+ T cells, thereby causing preferential proliferation of the CD4+ T cells as well as killing of CD8+ T cells [114]. Enrichment before stimulation is more reliable and typically results in higher yields and purities.

Effector CD8+ T cell preparation

As effector cells, three types of cells are commonly used:

- CD8+ T cell clones or cell lines
- Non-stimulated, resting CD8+ T cells
- Stimulated, expanded CD8+ T cells

Much of the research on VIA has been done using **CD8+ T cell lines or clones**. These are prepared by repeated HIV peptide stimulations in combination with a polyclonal stimulus and/or γ-chain cytokines (IL-2, IL-7, IL-15) to induce proliferation. Cell lines/clones are useful tools to perform in-depth studies on epitope specificity, functional avidity, transcription profiles or the influence of HLA restriction, but are less suited to study differences between different patients and the relation of VIA activity with clinical parameters. In addition, epitope-specific clones, which are kept in long-term *in vitro* cultures, use a single T cell receptor and might have less cross-reactivity than primary epitope-specific CD8+ T cell populations that are polyclonal [115].

Non-stimulated primary CD8+ T cells are either rested for the duration of the CD4+ T cell activation or kept frozen until the start of co-culture and are used to measure the "*ex vivo*" VIA of a patient's CD8+ T cells. To measure VIA after ***in vitro* stimulation/expansion** of the effector cells, CD8+ T cells can be stimulated in a non-specific, polyclonal way (e.g. bispecific monoclonal anti-CD3/CD4 antibodies in combination with IL-2 [108]) or in more specific ways, using HIV peptides or monocyte-derived dendritic cells (Mo-DCs) transfected with HIV mRNA or pulsed with peptides. As non-specific stimulation methods are usually strong and induce proliferation, few cells are needed and viral suppression is easily detectable. An important disadvantage, however, is higher "background inhibition" by T cells from non-infected subjects and a questionable *in vivo* relevance of the results. Relatively few studies have used the more specific stimulation methods, using pulsed Mo-DCs [116] and HIV peptides in combination with IL-2 [57, 117-120]. From our own experiments, we have observed that the use of Mo-DCs and peptides in combination with IL-2 also suffers from considerable background viral inhibition (unpublished data), likely due to the strong activation of CD8+ T cells by the

Mo-DCs and IL-2. We therefore recommend to stimulate effector cells with peptides and as little IL-2 as possible (maximum 10 U/mL), if feasible without IL-2 altogether.

It is important that effector cells are pure CD8+ T cells before being put in co-culture to avoid interference from other cell fractions (e.g. natural killer cells). Therefore, negative selection methods need to be used to remove CD8+ non-T cells. In addition, negative selection ensures that effector cells are not influenced in non-specific ways by binding to beads coupled to cognate antibodies.

Superinfection of CD4+ target T cells

After stimulation, activation and enrichment, CD4+ target cells are superinfected. While most studies have used lab-adapted viral strains, a few have used autologous viruses previously obtained from primary culture of CD4+ T cells [117, 121, 122]. Although VIA against autologous virus was observed to be slightly stronger for some patients, no consistent discrepancies have been reported with lab-adapted viral strains [121]. The choice between both will depend on the feasibility to obtain autologous virus and ultimately the research question to be answered. Using autologous virus is warranted for in-depth studies investigating e.g. escape mutations or epitope specific CD8+ T cells. The choice of lab-adapted viral strain depends on the subtype prevalence within the study population and the epitopes targeted by a vaccine. While some groups routinely use a C-C chemokine receptor type 5 (CCR5) tropic HIV-1 (such as BaL) in parallel with a C-X-C chemokine receptor type 4 (CXCR4) tropic HIV-1 strain (such as III_B) [83], this might not be necessary as an influence of HIV tropism on CD8+ T cell activity has not been reported yet. Besides, it has been shown multiple times that effective and clinically relevant CTL responses do not target the variable Env epitopes but rather the more conserved Gag and Pol epitopes [123-125].

The infection dose or multiplicity of infection (MOI) should be such that a sufficiently large amount of virus is produced at the peak of viral replication in order to allow discrimination between weak and strong CD8+ T cell responses. In his Nature Protocol paper Sáez-Cirión described that, when using p24 enzyme linked immunosorbent assay (ELISA) for the evaluation of suppression, between 100 and 1,000 ng/mL of p24 should be produced at the peak of viremia. Lower amounts do not allow discrimination between weak and strong responses, whereas higher viral replication is probably too strong even for the most potent CD8+ T cells. Similarly, when using intracellular *gag* staining, the ideal level of infection should be around 10 to 30% [109].

Infecting versus superinfecting virus

Since autologous CD4+ T cells are used from HIV+ patients, it is possible that autologous virus will replicate as well during the VIA assay besides the virus used for superinfection. However, in HIV+ patients on combination antiretroviral therapy (cART) with suppressed viremia, given the relatively short duration of culture and small amounts of (infected) CD4+ T cells, the probability of autologous virus emerging during the assay is rather small, and amounts of autologous viral replication are usually much smaller than that of the superinfecting virus. Nevertheless, it is good practice to include non-superinfected CD4+ T cells as controls to be able to correctly interpret the VIA data. Alternatively, a superinfecting virus which is resistant to an antiretroviral drug can be used, allowing the addition of this antiretroviral in the culture medium and blocking replication of any autologous virus [83, 126].

Co-culture of target and effector cells

Superinfected CD4+ target T cells are then put in co-culture with CD8+ effector T cells. This is usually done in medium containing relatively high amounts of IL-2 to keep the cell-cultures

alive [108, 109, 120]. From our experience, high amounts of IL-2 in the co-culture (as opposed to during the prior stimulation of effector cells) do not influence VIA as it does not increase background inhibition by effector CD8+ T cells from non-infected subjects (unpublished data).

The choice of effector to target ratio (E:T) will depend on the expected VIA. The literature describes suppression of viral replication at E:T ratios ranging from 5:1 to as low as 1:10 [111, 112]. Since it is hard to predict VIA beforehand, it is again good practice to test several E:T ratios in parallel.

Measurement of viral replication

Finally, viral inhibitory activity is calculated as the difference in viral replication between target cells alone and target cells in co-culture with effector cells. The percentage suppression is calculated as:

$$\%\ suppression = \frac{(Viral\ replication\ in\ target\ cells\ only) - (Viral\ replication\ in\ coculture)}{(Viral\ replication\ in\ target\ cells\ only)}$$

Viral replication can be measured by quantifying p24 in the supernatant with ELISA [107, 108, 127-129], by determining intracellular *gag* with flow cytometry [109, 130] or alternatively by infecting reporter TZM-Bl cells with VIA assay supernatants [131, 132]. Measuring p24 levels in the supernatant by ELISA is more sensitive and quantitative than measuring the number of infected cells with intracellular *gag* staining but is also more expensive and requires more cells [109]. A novel assay has been described by Naarding *et al.* where target cells are superinfected with an HIV-1 NL4.3 strain transformed with a *Renilla reniformis* luciferase reporter [110]. They report an increased sensitivity and specificity as well as a shorter assay time.

VIA in elite controllers versus chronic progressors

All studies comparing VIA in EC or (VC) versus CPs have observed much stronger VIA in controllers, be it in patients infected with HIV-1 or HIV-2 [133], who are treatment-naive or on cART [112] and who started treatment early or late [57, 112, 121, 131-135]. This difference is consistent whether or not effector cells are stimulated and regardless the type of autologous target cells: resting or activated CD4+ T cells or monocyte derived macrophages [113, 118]. Besides this remarkably stronger VIA, EC CD8+ T cells are characterized by more frequent polyfunctionality [136], sustained proliferative capacity [137] and frequent presence of the HLA-B*27 and B*57 alleles [138, 139] as compared to CPs.

Interestingly, the total number of HIV-specific CD8+ T cells is comparable between ECs and CPs off cART but is much higher than in suppressed CPs on cART [121]. Thus, CPs off treatment fail to control viremia despite their high numbers of CD8+ HIV specific T cells while CPs on treatment fail to sustain high numbers of HIV specific cells despite their similarly low viral loads and therefore antigenic exposure as ECs. Clearly, the ability to maintain high levels of circulating HIV specific CD8+ T cells in the face of very low peripheral viral load seems to be an intrinsic characteristic of ECs.

As a possible explanation, Migueles *et al.* established that ECs have a stronger per cell capacity to lyse target cells and respond better to peptide stimulation than CPs, as evidenced by higher proliferation and granzyme B (GrB) and perforin production [135]. Akinsiku *et al.* also found a correlation between polyfunctionality including IL-2 production and VIA in ECs [131] (Fig. 2A).

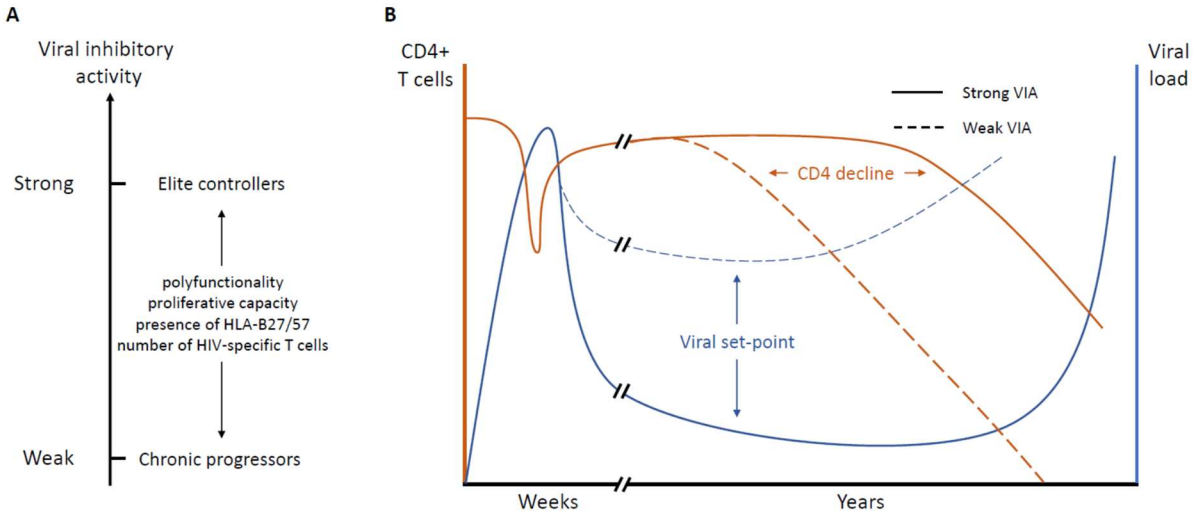

Figure 2. (**A**) Elite controllers (ECs) show much stronger viral inhibitory activity (VIA) than chronic progressors (CPs). At the same time, ECs are characterized by the presence of protective HLA alleles, higher numbers of circulating HIV-specific T cells, which also frequently show polyfunctionality and higher proliferative capacity as compared to CPs, possibly explaining the difference in VIA. (**B**) Stronger VIA in viremic patients off cART has been associated to slower CD4+ T cell decline and lower viral set-point.

Evidence of induction of VIA

Induction by *in vivo* vaccination

In the field of SIV infection, several groups have reported increased *ex vivo* VIA after *in vivo* prime/boost vaccination with or without subsequent viral challenge. VIA correlated with higher numbers of virus-specific CD8+ T cells [127] and inversely correlated with viral load (VL) peak and set-point [129]. Stephenson *et al.* confirmed this inverse correlation between VL set-point and VIA after vaccination, and in addition demonstrated that VIA correlated with *gag*-specific and not *pol/env*-specific responses [140]. Thus, VIA can be induced by *in vivo* vaccination, is correlated with *gag*-specific cellular immune responses and is clinically relevant as it correlates with viral control after break-through infection. In the field of HIV-1, a number of phase 1 vaccination trials, all using DNA prime/viral vector boost strategies, have similarly shown clear induction of VIA after vaccination [108, 119, 122, 141, 142]. As will be discussed in a

following chapter, the same *gag*-specificity and correlation with viral set-point mentioned above for SIV, has been reported for VIA in HIV as well [114, 121, 143].

Induction by *in vitro* stimulation

Robust *in vitro* models able to test the potential of vaccine candidates to induce *in vivo* VIA are needed but only limited data has been published so far. In 2001 Lu *et al.* described a model where Mo-DCs pulsed with inactivated virus and used to stimulate peripheral blood mononuclear cells (PBMCs) led to the expansion of HIV-specific cells able to kill HIV-infected cells [116]. Two more recent publications used HIV-1 *gag* peptide pools in combination with IL-2 to directly stimulate PBMCs, increasing the capacity of CD8+ T cells to kill infected CD4+ T cells [117, 118]. In recently published work from our own group, PBMCs specifically stimulated with an HIV-1 *gag* peptide pool but without the presence of any IL-2 caused an important upregulation of VIA in HIV+ patients on cART [120]. Finally, as it is known that latency reversing agents (LRA) can inhibit CD8+ T cell function, one group investigated and indeed confirmed the inhibition of VIA by LRAs such as bryostatin-1, prostratin and JQ1 [144].

Clinical correlates with VIA

As early as the year 2000 it was reported that weaker VIA predicted faster CD4+ T cell decline in therapy naive HIV+ patients [145]. More than ten years later, this was confirmed by a study which also observed an inverse correlation with the VL set-point [143], a finding which in turn was backed up by the results of an already mentioned study in vaccinated rhesus monkeys [140] (Fig. 2B).

These data indicate that strong CD8+ T cell VIA activity *in vitro* is associated with lowered *in vivo* viral replication, resulting in a low VL and a delayed decline of CD4+ T cell count. However, it can be hypothesized that sustained antigenic exposure is required for the maintenance of

virus-specific CD8+ T cell responses and strong VIA activity. This hypothesis is supported by a publication from Freel *et al.* showing that VIA declined over time with resolving VLs [132]. Interestingly, the study even showed comparable VIA activity in ECs and CPs early in infection, while it is known that in chronic infection VIA is much stronger in ECs than in CPs, as discussed previously. Spentzou *et al.* showed that viremic controllers have stronger VIA than patients on cART [108]. Noel *et al.* further showed that VIA is significantly stronger in ECs whose CD4+ T cells produce more virus after stimulation with latency reversing agents than ECs with weaker VIA responses [146]. Patients with more easily inducible proviruses are more likely to have occasional, residual viremia and antigenic exposure, presumably leading to continued boosting of the immune system and maintenance of potent CD8+ T cell responses, possibly explaining the above observations.

Certain protective HLA types (e.g. HLA-B*27, HLA-B*57) are enriched in ECs. Interestingly, ECs with such HLA-types also have the strongest VIA [112, 147]. In CPs, the image is less clear, as the presence of protective HLA types does not always lead to stronger VIA [114, 128]. In other words, there is no direct and straightforward relationship between HLA restriction and VIA.

Besides CD8+ T cell effector potency, the susceptibility of infected CD4+ T cell targets could also play a role. Buzon *et al.* observed that CD4+ T cells of ECs are more susceptible to cytotoxic T cell (CTL) killing than CD4+ T cells of CPs on cART [148]. Remarkably, this susceptibility was consistently higher in HLA-B*57 positive patients. Patients with higher CD4+ T cell susceptibility also had the smallest viral reservoir sizes.

In this regard a very intriguing observation was made by Huang *et al.* on an apparently inherent resistance to CTL killing of CD4+ T cells containing intact pro-viruses [149]. In this study, resting CD4+ T cells were treated with a combination of latency reversing agents and

autologous CD8+ T cells. While the total amount of pro-viral DNA was reduced, the replication competent fraction was not, indicating that only CD4+ T cells containing incomplete pro-viruses were being eliminated. Further research is needed to understand whether this preferential elimination is due to *nef* mediated downregulation of MHC I or due to another as yet unidentified inherent resistance mechanism of CD4+ T cells containing intact pro-viruses.

Correlations with cytokine production

The most studied cytokine in relation to cell cytotoxicity is interferon-γ (IFN-γ). It is routinely measured in ELISpot assays to determine the frequency of HIV-specific cells, the antigen specificity, as well as the breadth and avidity of the immune response. A study from Sáez-Cirión *et al.* observed a strong correlation between the frequency of IFN-γ producing T cells and VIA in ECs [121]. Another study illustrated the variability within epitope specificities, showing a correlation between VIA and numbers of IFN-γ producing cells after *env* but not after *gag* stimulation [128]. Yet another study nicely showed that the breadth, and not the magnitude, of *gag* responses is a determining factor for VIA [114].

Interestingly, a study using CD8+ T cell lines specific for various gag epitopes observed 1000-fold differences in VIA between cell lines despite having comparable activity in IFN-γ ELISpot [128]. This suggests that correlations with IFN-γ are possibly confounded by the production of other bio-molecules responsible for viral suppression. In this regard, Freel *et al.* delivered the most convincing data. In their DNA prime/boost vaccination study, VIA was associated with cells co-expressing CD107a, MIP-1α and IFN-γ [122]. Nevertheless, VIA was only associated independently with CD107a and MIP-1α expression and not with IFN-γ, strongly suggesting that associations of IFN-γ with VIA are a consequence of the frequent co-expression of this and other cytokines by cells with real suppressive activity.

While the causal relationship between IFN-γ production and suppressive activity is dubious, the evidence for CD107a, a marker of degranulation on CD8+ T and NK cells, is more convincing. Several independent studies, both cross-sectional as well as prospective vaccination trials, in HIV as well as SIV models, have linked CD107a expression to *in vitro* VIA [122, 129, 150]. Thus, the capacity to deliver lytic granules seems to be predictive of *in vitro* VIA. Migueles *et al.* previously showed that lytic granule content is strongly associated with killing capacity, observing increases in perforin and granzyme B expression in response to peptide stimulation in HIV controllers and not ~~so~~ in progressors [135]. The importance of perforin was later confirmed [129]. Besides these classical cytotoxicity markers, a number of other cytokines have been linked to viral suppression, such as IL-2, MIP-1α, MIP-1β and TNF-α [150].

Taken together, the evidence suggests that VIA cannot be predicted by the expression of one marker only. Rather, the ability to produce several cytokines simultaneously and to degranulate with high perforin and grB content in response to cognate antigen stimulation seem necessary to achieve potent suppression of viral replication.

Epitope specificity and avidity

Specificity

T cell responses to different parts of the HIV proteome have been associated with variable levels of protection against disease progression. Responses to *gag* peptides have most often been correlated to lower viral loads [123-125], but so have responses to *pol* and *vif* [83, 119, 151] and even to some *env* peptides [83, 152]. These differences are also reflected in associations between T cell specificity and VIA. In general, CD8+ T cells or clones targeting *gag* epitopes display the strongest VIA, but responses specific for *pol, tat* and *nef* have also been reported to be suppressive [83, 114, 121, 128, 130, 151, 153, 154]. More precisely, while there is no correlation with

the breadth or magnitude of bulk CD8+ T cells [108], the breadth of *gag* specific responses is associated with VIA, polyfunctionality and even reduced *in vivo* viral loads [114, 121, 147, 155]. As with *in vivo* control, *gag* responses thus seem to be crucial for *in vitro* viral suppression, while (most) *env* responses are not. Presumably, *gag* peptides from incoming virions can be presented on HLA molecules within hours after infection, while *env* peptides first need to go through synthesis and processing and are only presented 24 hours after entry at the earliest [156-158]. Besides this rapid antigen presentation of *gag* peptides after infection, the role of other determining factors such as protein expression levels, amino acid composition, processability, immunogenicity and escape potential need further investigation.

Concerning the importance of antigen specificity for VIA, Pohlmeyer *et al.* made two interesting observations. They observed that ECs were able to suppress viral replication of both wild type NL4.3 virus as well as mutants containing escape mutations in HLA-B*57 restricted *gag* epitopes [159]. No CPs were included in these experiments, so it is unknown whether the recognition of escape mutants distinguishes ECs from CPs. Nevertheless, another study showed that a single amino acid difference can significantly change epitope binding avidity and strongly affect VIA in CPs [160]. It is therefore unlikely that VIA in CPs is very forgiving for escape mutations, while this might be the case in ECs.

Interestingly, Pohlmeyer *et al.* also demonstrated that microbial peptides can cross-react with HIV-specific T cells and induce VIA against HIV-1 [161]. The T cell receptor diversity was shaped differently in different patients in response to the same microbial peptide pool, indicating that anti-HIV immunity can be modulated by non-HIV, microbial peptides.

Avidity

Avidity is commonly defined as the peptide concentration which elicits half maximal response rates in assays such as IFN-γ ELISpot or ^{51}Cr release, i.e. the lower this concentration, the higher the avidity. T cell responses directed at protective epitopes have higher avidity in ECs than in CPs [126]. In the same way, avidity of T cell responses has been positively correlated with VIA [83, 128, 160, 162-166]. As an example, Bennett *et al.* nicely illustrated how avidity could explain the discrepancy between cross-reactivity in ^{51}Cr release assays and VIA [160]. While responses against several peptides were detected in the former assay (which utilizes supra physiological peptide concentrations), not all of these responses proved to cause suppression in the latter assay (where peptides are presented by target cells at lower concentrations). Only responses with high avidity were able to suppress viral replication. Furthermore, they determined that the relationship between avidity and VIA follows a sigmoidal curve, where suppressive activity quickly drops below a certain avidity threshold (Fig. 3).

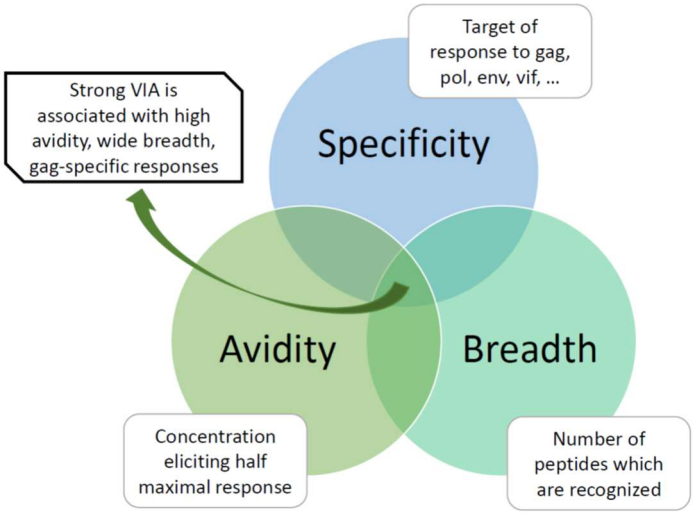

Figure 3. In terms of T-cell responses, strong viral inhibitory activity (VIA) has been separately associated with the capacity to recognize a wide range of epitopes (breadth), a response mainly targeting gag-epitopes (specificity) with strong functional affinity of the T-cell receptor for the peptide (avidity). This suggests that T-cell responses corresponding to these three criteria will show strongest VIA.

Despite the clear association between avidity and viral suppressive activity, the following nuance must be made. Chen *et al.* and Lissina *et al.* both found that (1) T cell responses against *gag* resulted in stronger VIA than responses against *env,* and (2) responses against *gag* had higher avidity than responses against *env* [128, 165]. Nevertheless, when *gag* and *env* responses were analyzed separately, no correlation was observed anymore between avidity and VIA. At the same time, other studies have established a correlation between VIA and avidity within the same epitope specificity and as a result, it remains under debate whether epitope specificity is more important than avidity in determining VIA.

Remarkably, high avidity responses have been linked both to higher[162] as well as lower polyfunctionality[167]. On the one hand, strong binding of the cognate antigen to the T cell receptor might more easily induce activation cascades, leading to polyfunctional cells. On the other hand, high avidity will also lead to stronger expansion and higher turnover of the stimulated cells, which might negatively affect their lifespan and lead to irreversible exhaustion [168, 169].

Differentiation stage and phenotype

Terminally differentiated CD8+ T cells directed against HIV-1 are more frequent in ECs than in CPs [170]. In addition, two independent papers on HIV and SIV have reported that VIA can be measured in all effector and memory T cell subsets, except for naïve T cells. In a study by Julg *et al.* where broad *gag* responses were correlated with stronger VIA, these same broad *gag* responses were also correlated with higher numbers of terminally differentiated RA^+ effector memory T cells (T_{EMRA}) as a percentage of bulk CD8+ T cells. Interestingly, Jensen *et al.* found that VIA was correlated with higher CD57 expression on effector T cells and higher PD1/2B4/CD160 co-expression on effector memory T cells (T_{EM}). CD57 is a marker of terminal

differentiation and senescence, while PD1/2B4/CD160 are all activation/exhaustion markers. In our own research we have observed similar findings, with increased CD57 expression in sub clusters of T_{EM} and T_{EMRA} cells as well as increased co-expression of PD1/CD160 in sub clusters of T_{EMRA} cells in patients with high VIA [120]. Even though the evidence base is still small, taken together, it seems that cells responsible for suppressing viral replication tend towards more activated and (terminally) differentiated phenotypes. Such cells may be more likely to quickly mediate cytolytic activities than earlier memory phenotypes, although they also might be shorter lived.

Finally, ECs also have higher HLA-DR expression on CD8+ T cells than patients on cART [112]. This HLA-DR^{high} profile has been associated to stronger VIA [121]. In our own work we have observed a similar trend, with higher HLA-DR expression in CPs with higher VIA [120]. HLA-DR is a typical cell activation marker, but is also associated with proliferation [171]. Consistent with reports on higher proliferative capacity of HIV specific T cells in ECs, this higher HLA-DR expression on CD8+ T cells showing strong VIA might reflect the proliferative potential of these cells.

Conclusions

It is becoming increasingly clear that a functional cure for HIV-1 cannot be achieved by merely reducing the size of the viral reservoir. A series of treatment interruption studies including patients with extremely small viral reservoirs have had disappointing outcomes, with hardly any delayed viral rebound [53, 67, 172-175]. Immune interventions targeting cytotoxic CD8+ T cells will most likely be necessary to achieve sustained suppression of viremia in the absence of cART. However, a number of therapeutic vaccination trials have been equally unsuccessful in achieving *in vivo* control of viremia, even though classic immune parameters such as IFN-γ

ELISpot and ICS provided evidence of vaccine immunogenicity. Nevertheless, it is believed that therapeutic vaccination still has a lot of potential, attributing the failure of previous trials to flawed immunogen design, rather than a flawed strategy altogether. Clearly, an assay able to predict *in vivo* viral control is needed to evaluate therapeutic vaccination candidates before going into clinical trials.

In vitro VIA assays have been convincingly associated with CD4+ T cell count maintenance, *in vivo* viral suppression, polyfunctionality and the cytotoxic potential of CD8+ T cells (Fig. 4). There is ample evidence that VIA can be induced *in vivo* and we even know which antigenic targets have the most potential in terms of protection. Today, research is needed to determine whether *in vitro* VIA can actually predict *in vivo* viral remission or not.

VIA assays are challenging to set-up, are labor intensive and take several weeks to finish. In addition, due to their complexity, a lot of different set-ups have been published, making comparisons in between studies less straight-forward as compared to e.g. IFN-γ ELISpot results. In short, VIA assays are not the easiest, cheapest or fastest, but they should be studied further in future prospective treatment interruption trials.

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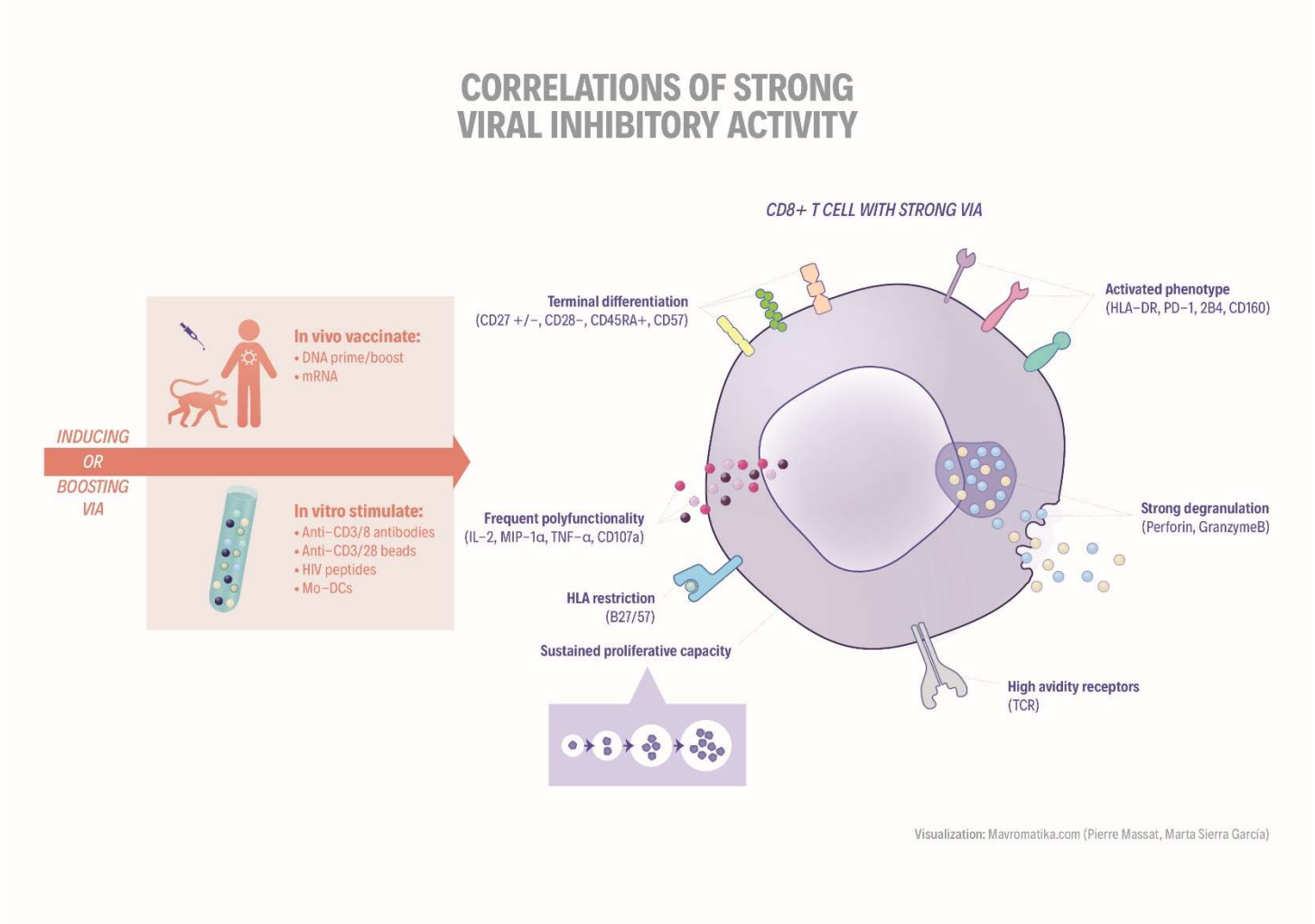


Figure 4: Schematic summary of the most important characteristics of CD8+ T cells that have been associated to viral inhibitory activity (VIA) as well as a number of evidenced ways to induce or boost VIA *in vivo* and *in vitro*.

Chapter 3 - Scope and outline of thesis

Rationale

Worldwide millions of people are infected with the human immunodeficiency virus (HIV), the causative agent of the acquired immune deficiency syndrome (AIDS). While currently available combination antiretroviral therapy (cART) effectively blocks HIV replication, HIV persists in a reservoir of latently infected CD4+ T cells [39, 176]. As a result, when cART is interrupted, most patients experience viral rebound within two to four weeks [177]. Only a small proportion of patients has been described to control viral replication without treatment after a period of cART, a phenomenon termed "post treatment control" or PTC [55, 178]. A recent review defines such PTCs as patients maintaining viral loads below 400 cps/mL for at least 24 weeks after treatment interruption. This PTC status effectively corresponds to a 'functional cure', where a patient is still infected but disease free without the need of further treatment. The factors that lead to this PTC status are as yet unknown.

The research project presented in this thesis is based on the hypothesis that such a functional cure of HIV infection can be achieved when two conditions are met:

1. the size of the viral reservoir is sufficiently reduced.
2. the functioning of the host's immune system is sufficiently restored.

While it is not clear yet how the **size of the reservoir** is best quantified, different reservoir measures inversely correlate with time to viral rebound in cART patients who interrupted their treatment. Indeed, the amount of total HIV DNA, integrated DNA and cell-associated unspliced RNA (measured with PCR technologies) as well as the replication competent fraction (measured with quantitative viral outgrowth assays) have all been associated with time to rebound [54, 179-183]. As would be expected, the smaller the reservoir measure, the longer it takes for a patient to experience viral rebound.

This size of the reservoir depends on and can be influenced by a number of factors. The earlier treatment is started after HIV infection and the longer patients have been on treatment, the smaller the patient's viral reservoir will be [184]. This is one of the reasons why today, newly infected people are started on therapy immediately after diagnosis. In addition, so-called "shock-and-kill" strategies aim to reactivate latent pro-viruses (shock) to make infected cells visible to the immune system so that they can be eliminated (kill), thereby reducing the size of the reservoir.

In terms of **immune function**, there is strong evidence that CD8+ T cells play a major role in the control of viremia during infection, as discussed in the introduction of this thesis. A wide variety of parameters exist that assess CD8+ T cell function, including proliferation, IFN-γ production and release in response to cognate antigen stimulation, polyfunctionality of individual cells, cytotoxic potential, epitope-specificity and avidity, differentiation profiles, exhaustion and senescence states, viral inhibitory activity, etc. However, none of these parameters have been directly correlated with *in vivo* control of viremia after treatment interruption. Nevertheless, indirect evidence of associations exist, as HIV+ patients who naturally control the infection typically score better for most of the listed parameters than HIV+ patients who show a rapid progression of the disease.

Based on these observations, different **strategies to improve immune function** have been proposed. The most important one is therapeutic vaccination, aiming to re-educate the host immune response in order to better control viral replication in the absence of antiretroviral therapy. Another strategy, which can complement a vaccine, is the use of immune checkpoint inhibitors to reverse the exhausted state of CD8+ T cells, a phenomenon often observed in

chronic infections. Correctly stimulated and reinvigorated CD8+ T cells are then hypothesized to be able to (better) control viral replication.

While the size of the viral reservoir has been associated with time to rebound, the association is not strong enough to be able to predict PTC after treatment interruption. Nevertheless, it seems straightforward that a small viral reservoir will increase the chances of success of any therapeutic intervention. The smaller the reservoir, the smaller the amount of infected cells that can reactivate, and the smaller the amount of target cells that need to be killed by the vaccinated effector CD8+ T cells.

In other words, the **central hypothesis** of this research states that the combination of a small reservoir and a recapacitated immune system can lead to viral remission after treatment interruption.

Objectives

In the search for an intervention that leads to functional cure, it is crucial to first understand what factors are predictive of and can explain this state of PTC. Therefore, the primary objective of this research project was to identify biomarkers that are predictive of PTC.

Thesis outline

Chapter four describes the findings from an analytical treatment interruption study (ISALA) in patients with low viral reservoir and proviral transcriptional activity. **Chapter five** reports on the results of a cross-sectional study which investigated possible correlations between VIA of CD8+ T cells and a number of clinical, immunologic and viral parameters in a population of virally suppressed HIV-infected individuals on cART. In **chapter six**, the results are reported of a therapeutic vaccination trial, where an innovative vaccination strategy was tested with the goal to achieve delayed viral rebound. Finally, **chapter seven** consists of a general discussion

on the presented data, our view on the future of finding a cure for HIV and a more general discussion of priorities in the global response against HIV/AIDS.

Chapter 4 - Rapid viral rebound after treatment interruption in patients with a very small viral reservoir and limited viral transcriptional activity

Author's contributions: The following chapter is entirely the author's work, except for the viral reservoir measurements (integrated DNA, t-DNA, US-RNA and LTR-RNA) by droplet digital PCR and the sequencing data obtained through single genome analysis which were both performed at the HIV Cure Research Center of the partner institution University Hospital of Ghent.

Abstract

Low levels of HIV-1 proviral DNA and cell-associated RNA have separately been associated to post treatment control (PTC) of viremia. We investigated whether the combination of both parameters could help predict delayed viral rebound after treatment interruption (TI).

We conducted an open single arm analytical treatment interruption study in four Belgian HIV reference centres. Eligible participants were adults who had fewer than 50 HIV-1 RNA copies/mL for more than two years, more than 500 CD4 cells per µL for more than three months, and were in general good health. Consenting participants who had fewer than 66 copies total HIV-1 DNA (t-DNA) and fewer than 10 copies cell-associated HIV-1 unspliced RNA (US-RNA) per million peripheral blood mononuclear cells (PBMCs), interrupted therapy and were monitored closely. Antiretroviral therapy (ART) was resumed after two consecutive viral loads exceeding 1,000 copies or one exceeding 10,000 copies/mL. The primary outcome was the proportion of participants with fewer than 50 HIV-1 RNA copies/mL 48 weeks after TI. Secondary outcomes were time to viral rebound, the frequency of serious adverse events, and evolution of t-DNA and US-RNA after TI.

All sixteen consenting participants who interrupted therapy experienced rapid viral rebound two to eight weeks after TI. No serious adverse events were observed. Levels of t-DNA and US-RNA increased after TI but returned to baseline after treatment restart.

In conclusion, the combination of low levels of t-DNA and US-RNA in PBMCs, corresponding respectively to a small and transcriptionally silent viral reservoir, is not predictive of viral remission after TI in patients on ART. Presumably, PTC is not only the result of a reduced viral reservoir size, but also of a sufficiently recovered immune response.

Introduction

Although antiretroviral therapy (ART) effectively blocks HIV replication, HIV persists in a reservoir of latently infected CD4+ T cells [39]. This causes most patients to experience viral rebound within weeks after treatment interruption [185]. Viral remission after analytical treatment interruption (ATI), termed post-treatment control (PTC), has been described in a small proportion of patients [55, 178, 186]. Although PTC has been associated with early treatment initiation [187], we and others showed that some PTCs initiated ART in the chronic phase [55, 188]. A single common characteristic of all well-documented PTCs is the presence of a very small reservoir similar to "elite" controllers [54, 179-181, 183].

Besides the size of the viral reservoir (measured as total HIV-1 cell-associated DNA or t-DNA), its transcriptional activity (measured as HIV-1 cell-associated unspliced RNA or US-RNA) also independently correlates with delay of viral rebound after ATI [179, 181, 189]. Indeed, a pooled analysis of six AIDS Clinical Trials Group ATI studies aiming at identifying predictors of viral rebound, revealed that higher levels of US-RNA are associated with shorter time to viral rebound (TTVR) after ATI [181]. However, no prospective studies have investigated the association between TTVR and the combination of a very small latent viral reservoir (t-DNA) and minimal on-going viral transcription (US-RNA) before ATI.

We therefore designed a single arm open ATI trial where patients on ART were selected based on both low levels of t-DNA and US-RNA. The aim of the study was to evaluate whether these combined selection criteria could help to better predict delayed viral rebound.

The primary objective of this study was to determine the proportion of participants with plasma viral load <50 copies/mL at 48 weeks after ATI. Secondary objectives included a safety assessment of the ATI, TTVR, kinetics of plasma viral load as well as evolution of viral reservoir

t-DNA and US-RNA during ATI and after restarting ART. Exploratory objectives included the identification of other potential factors predictive of TTVR, such as demographic, clinical and biological parameters, including CD4+ T cell nadir, ultrasensitive plasma viral load (usVL) [190], integrated DNA and HIV-1 cell-associated LTR-RNA levels [191] as well as *in vitro* assays for viral RNA release [192] and infectious viral outgrowth [193]. Finally, we investigated the origin of rebound viruses by comparing *in vivo* rebound with *in vitro* outgrowth viruses by single genome analysis (SGA).

We observed rapid viral rebound after treatment interruption in all study participants, indicating that the combination of a small viral reservoir size (t-DNA) and low proviral transcriptional activity (US-NA) is not predictive of delayed viral rebound.

Methods

Study design and participants

In a first stage 114 consenting individuals were recruited from a total patient population of 7,212 HIV-1 positive people on ART from four major Belgian HIV Reference Centres: one in Antwerp (ITM), 2 in Brussels (UMC and UZB), and one in Ghent (UZG). Inclusion criteria included nadir CD4+ T cell count >300 cells/µL, current CD4+ T cell count ≥500 cells/µL for ≥3 months and plasma viral load (pVL) <50 copies/mL for ≥2 years. Exclusion criteria included, amongst others, pregnancy, breastfeeding, active hepatitis B or C infection and any psychiatric or psychological disorder that could interfere with participation in an ATI study.

Stage 1 participants were screened for t-DNA and US-RNA levels. Participants with t-DNA <66 copies per million peripheral blood mononuclear cells (PBMCs) and US-RNA <10 copies per million PBMCs were eligible for stage 2. The threshold for t-DNA was based on DNA levels from a previous clinical study [194]. The 15th percentile of the cohort was used as a threshold to

include only patients with a small reservoir. The threshold for US-RNA corresponded to the limit of detection of the assay when performed in three replicate reactions.

Consenting participants eligible for stage 2 were psychologically examined to evaluate their motivation for participation and to avoid potential misconceptions on study objectives [195]. A leukapheresis was performed and ART was interrupted (baseline) after sexual counselling. Participants were followed for 48 weeks or until ART resumption criteria were reached. In line with other treatment interruption studies [186], these criteria included two consecutive viral loads >1,000 copies/mL measured at least three days apart, a single viral load >10,000 copies/mL, CD4+ T cell count drop to less than 350 cells/µL at two consecutive measurements at least 2 weeks apart or a drop of more than 50% compared to baseline, any new significant clinical condition, a new sexually transmitted infection, pregnancy or participant withdrawal from the study.

On weeks 2/4/6/8/12/16/20/24/32/40/48 post ATI as well as week 4 and 12 post ART restart, whole blood was collected for plasma and PBMC separation (Fig. 1A). Plasma was used for immediate pVL measurement and the remainder was stored at -80°C. All PBMCs were stored in liquid nitrogen.

The study was approved by the internal review board of the Institute of Tropical Medicine (ITM) in Antwerp, the ethics committees of the University Hospitals of Antwerp (UZA), Ghent (UZGent) and Brussels (CHU St. Pierre, UZB) (respective approval numbers: 1012/15, 15/31/321, 2015/0771, AK/15-07-84/4539 and 2015/261) and was registered on clinicaltrials.gov with the code NCT02590354.

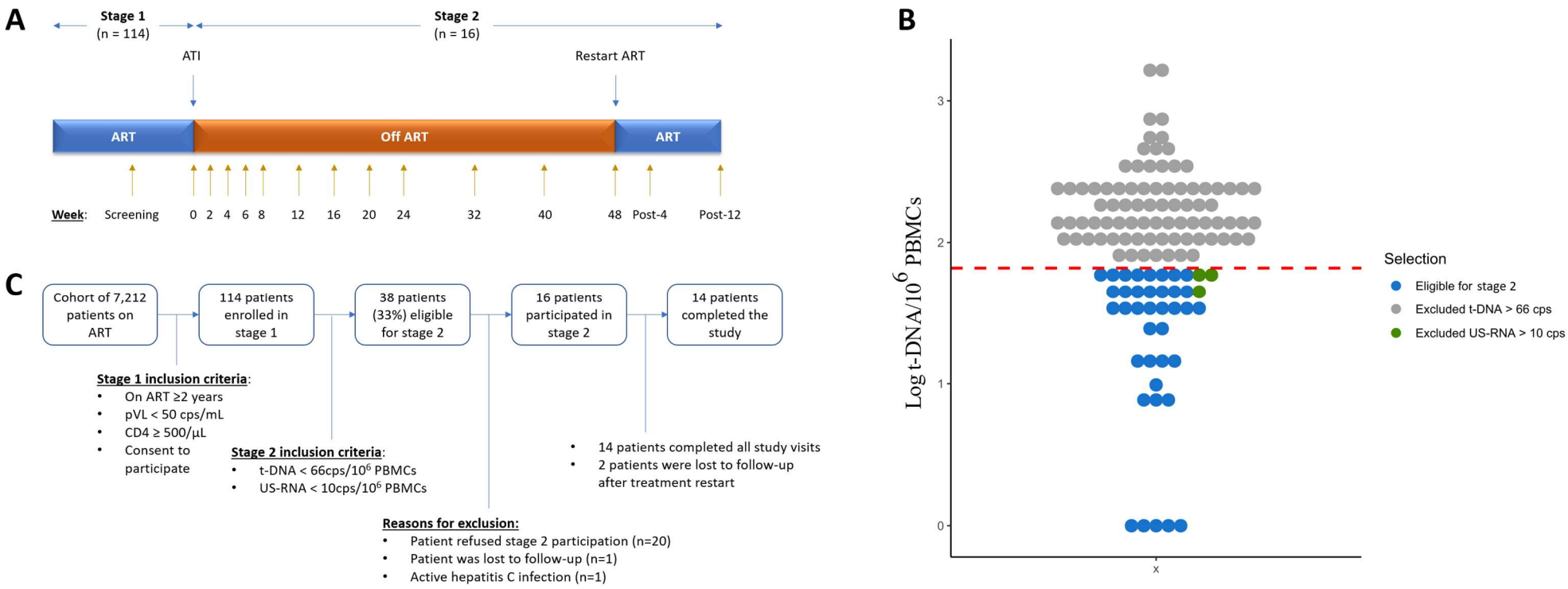


Figure 1. Study timeline and participant selection. (**A**) Overview of stage 1 and 2 of the study. Timepoints at which blood was drawn for plasma and PBMC separation are indicated with yellow arrows. ART is restarted when treatment resumption criteria are reached or 48 weeks after treatment interruption. (**B**) Total HIV-1 DNA (t-DNA) values in copies per million PBMCs for all stage 1 participants. Participants with t-DNA values >66 copies per million PBMCs are indicated with gray dots (n=75). Patients with t-DNA values below 66 copies but US-RNA values above 10 copies per million PBMCs are indicated with green dots (n=3). Patients with t-DNA and US-RNA values below 66 and 10 copies per million PBMCs respectively are indicated with blue dots (n=38) and are eligible for stage 2 participation. (**C**) Flow chart with number of patients screened until number of patients who completed the study (ATI = analytical treatment interruption; t-DNA = Total HIV-1 DNA; US-RNA = cell-associated HIV-1 unspliced RNA).

Safety assessments

Safety assessments were performed at each study visit and included evaluation of clinical signs as well as laboratory parameters. The severity of adverse events (AEs) was scored according to the Division of AIDS toxicity table [196].

Cell-associated HIV-1 DNA and RNA measurements

At screening, t-DNA and US-RNA were measured by droplet digital polymerase chain reaction (ddPCR). Subsequently, in the selected stage 2 participants, the following HIV-1 measurements were performed: t-DNA and cell-associated LTR HIV-1 RNA (LTR-RNA) in PBMCs at baseline, weeks 2/4/6/8/12/16/20/24/32/40/48 post-ATI, and week 12 post-ART restart (PW12); integrated HIV-1 DNA in PBMCs at baseline, before ART restart and PW12; LTR-RNA in CD4+ T cells at baseline (Fig. 1A). US-RNA targets the gag/pol region and represents a late complete transcript, whereas LTR-RNA targets the 5'-LTR region, representing the earliest incomplete transcript [191, 197]. For technical details see S1 methods.

Ultra-sensitive plasma viral load (usVL)

UsVL was measured as described by Leal *et al.* with minor modifications [190]. In short, 10mL of EDTA plasma was concentrated to 0.8mL by ultracentrifugation at 170,000 x g for 1h in a swinging bucket rotor (Beckman Coulter, Brea, CA, USA) and tested on the Roche Cobas® 4800 system. UsVL results were multiplied by 0.08 (0.8/10) to account for the concentration. The assay can reliably detect viral loads ≥5 copies/mL.

Quantitative viral outgrowth assay (qVOA)

The qVOA was performed on 24 x 10^6 CD4+ T cells as described by Laird *et al.* with some modifications [193]. For technical details see S2 methods.

Viral release assay (VRA)

The VRA was performed as described by Cillo *et al.* with minor modifications [192]. PBMCs and CD4+ T cells were cultured in unstimulating and stimulating conditions. The unstimulated condition refers to overnight resting in RPMI 10% FCS + 0.6% penicillin/streptomycin + 100nM Dapivirine + 100nM Raltegravir. For the stimulated condition, cells were incubated for 7 days in the same complete medium with additional 50ng/ml phorbol myristate acetate (Sigma, Diegem, Belgium) and 500ng/ml ionomycin (Sigma). PBMCs were plated in 6-well plates at 15 x 10^6 cells per well in 5mL medium. CD4+ T cells were plated in 24-well plates at 5 x 10^6 cells per well in 2.5mL medium. Supernatant was stored at -80°C until assessment of virus release by quantitative real-time PCR with the commercial COBAS HIV-1 viral load test (Roche, Basel, Switzerland).

Single-Genome Analysis (SGA) comparing viruses from qVOA and rebound plasma

Supernatant from TZM-Bl-positive qVOA wells from 3 participants (1011, 4012 and 3033) with more than 2 positive qVOA wells (resp. 13, 11 and 3 wells) was harvested and 10µL was subjected to RNA extraction using the QIAamp mini viral RNA kit (Qiagen), according to manufacturer's instructions. Viral RNA was extracted from plasma samples at time of rebound, as described previously [198]. Subsequently, cDNA was generated using the Superscript III RT kit (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. In the case of rebound samples, a dilution series (1:3 to 1:243) of the cDNA was made, aiming for ≤30% positive reactions, and the V1-V3 env region (894 bp) was amplified using a nested PCR, as described previously [199]. For qVOA samples, five replicates of the same nested PCR were performed on bulk cDNA, to ensure the presence of only a single virus per positive well. Positivity of reactions was checked on a 1% agarose gel, and amplicons were sequenced from both ends by Sanger sequencing, using second round primers.

Phylogenetic analyses

Phylogenetic analyses on V1-V3 sequences derived from qVOA wells and plasma were performed as described previously [199, 200]. Sequences that did not meet quality standards and hypermutated sequences were omitted from further analysis. The remaining sequences were used to construct contigs, which were aligned with MUSCLE [201]. In the case of qVOA sequences, the five replicate contigs per positive well were multiple aligned and the consensus sequence was extracted. Maximum likelihood phylogenetic trees were constructed with PhyML v3.1 [202], using the general time-reversible (GTR) nucleotide substitution model with 1,000 bootstraps. Additionally, a neighbour-joining tree with all sequences from the three subjects was created to check for inter-sample contamination.

Statistical analysis

Categorical data are presented as frequency counts and percentages, and continuous variables are presented as medians and interquartile ranges. Continuous measurements were compared using the Wilcoxon signed rank and Mann-Whitney test in case of dependent and independent samples respectively. Spearman rank correlation coefficients were calculated to determine correlations between VRA and US-RNA results. To analyse virologic markers over time a Friedman Rank Sum test, followed by a post-hoc Nemenyi test for unreplicated blocked data, was performed on participant samples without missing data for baseline, timepoint before restart ART and 12 weeks post-ATI. Cox proportional hazard models were used to calculate the probability of viral rebound at each time point and to assess associations of clinical, virologic and immunological parameters with TTVR dynamics.

Results

Participant selection and clinical characteristics

In stage 1, 114 consenting participants were recruited (characteristics in S1 Table). Median t-DNA and US-RNA levels were 107 [IQR: 46–205] and 3.0 [IQR: 1.0–6.0] copies per million PBMCs, respectively. Forty-one (36.0%) had t-DNA levels <66 copies per million PBMCs. Of those, thirty-eight (92.7%) also had US-RNA levels <10 copies per million PBMCs and were therefore eligible for treatment interruption (Fig. 1B). Twenty-two did not consent or were excluded for participation in stage 2 for various reasons (Fig. 1C). The 16 remaining stage 2 participants included 15 men and one woman with median age 44 years [IQR: 38–54], median time on ART 4.0 years [IQR: 2.9–6.2] and median CD4+ T cell count 758 cells/μL [IQR: 679–845]. Time since diagnosis was significantly shorter in the 16 patients who participated in stage 2 (median [IQR]: 3.9 years [2.8-6.3]) as compared to the patients who were not eligible for stage 2 (median [IQR]: 6.2 years [4.0-9.6]). Detailed participant characteristics are given in table 1.

Treatment interruption with close follow-up is safe

ATI was generally safe as no serious AEs were recorded. However, most participants (13/16; 81%) reported an AE, with a total of 32 AEs recorded. All AEs were mild or moderate in intensity and resolved by the end of the study period. No side effects specifically related to the leukapheresis were reported. Three AEs (in 3 participants, 18.8%) were deemed to be related to the treatment interruption, occurred around the time of viral rebound and resolved spontaneously (S2 Table).

S1 Table. **Characteristics of study participants.**

	Enrolled in phase 1 (n=114)	**Non eligible for phase 2 (n=75)**	**Eligible for phase 2 (n=39)**	**Enrolled in phase 2 (n=16)**
Age (years)	45.5 (38.0 - 52.0)	45.0 (38.0 - 52.0)	46.0 (38.0 - 54.0)	43.5 (38.0 - 54.0)
Female sex	6 (5.3%)	4 (5.3%)	2 (5.1%)	1 (6.3%)
Ethnicity				
White	111 (97.4%)	72 (96.0%)	39 (100%)	16 (100%)
Other	3 (2.6%)	3 (4.0%)	0	0
HIV risk group				
Men who have sex with men	95 (83.3%)	63 (84.0%)	32 (82.1%)	12 (75.0%)
Heterosexuals	14 (12.3%)	8 (10.7%)	6 (15.4%)	3 (18.8%)
Unknown	4 (3.5%)	3 (4.0%)	1 (2.6%)	1 (6.3%)
Transfusion, non-hemophilia related	1 (0.9%)	1 (1.3%)	0	0
Time since HIV diagnosis (years)	5.9 (3.9 - 8.4)	6.2 (4.0 - 9.6)	4.9 (3.2 - 8.1)	3.9 (2.8 - 6.3)
Time on cART (years)	4.3 (3.0 - 6.0)	4.4 (3.1 - 5.9)	4.3 (2.8 - 6.6)	4.0 (2.9 - 6.2)
Last used regimen				
II + NRTI	49 (43.0%)	30 (40.0%)	19 (48.7%)	11 (68.8%)
NNRTI + NRTI	51 (44.7%)	38 (50.7%)	13 (33.3%)	4 (25.0%)
PI + NRTI	4 (3.5%)	2 (2.7%)	2 (5.1%)	0
Other	10 (8.8%)	5 (6.7%)	5 (12.8%)	1 (6.3%)
HIV subtype B infected	106 (93.0%)	70 (93.3%)	36 (92.3%)	15 (93.8%)
Nadir CD4 (cells/µL)	379.0 (338.0 - 482.0)	367.5 (330.0 - 475.0)	430.0 (350.0 - 506.0)	440.5 (342.0 - 500.5)
CD4 count (cells/µL)	733.0 (645.0 - 891.0)	737.0 (645.0 - 912.0)	726.0 (644.0 - 854.0)	758.0 (679.0 - 845.0)

Data are median (IQR) or n (%). cART=combination antiretroviral therapy. II = integrase inhibitor. NRTI=nucleoside reverse transcriptase inhibitor. NNRTI=non-nucleoside reverse transcriptase inhibitor. PI=protease inhibitor.

Table 1. Individual characteristics of stage 2 study participants (n=16).

Study ID	Time on ART (years)	Time with undetectable VL (years)	CD4 nadir (cells/µL)
1011	3.2	2.9	446
1029	3.0	2.8	661
1038	4.7	4.2	379
1043	6.9	3.4	435
1044	11.4	7.1	328
2007	2.6	2.5	506
2022	2.6	2.5	302
2025	6.4	5.5	402
3024	15.6	8.5	467
3033	7.8	6.8	319
3034	3.0	2.4	356
4002	4.3	4.0	454
4006	3.5	3.1	495
4010	2.7	2.6	554
4012	4.7	4.4	303
4018	3.7	3.4	573

Rapid viral rebound following treatment interruption, complete resuppression of viremia and normalization of reservoir measures after treatment restart

All participants experienced viral rebound between 2 and 8 weeks post ATI (Figs 2A and S1). All but one participant resumed ART immediately after reaching viral rebound criteria. Participant 4012 reached rebound criteria by week 8 but maintained pVLs <3,500 copies/mL until week 11, when ART was restarted. By 12 weeks after ART restart, all participants in follow-up suppressed viremia again below 50 copies/mL. Two participants were lost to follow up after treatment restart. One restarted treatment shortly after the viral rebound and returned to follow-up 11 months later with an undetectable viral load. The other one had had an undetectable viral load four weeks after restart but moved to another country afterwards.

Total and integrated DNA and LTR-RNA were determined before and during ATI as well as after restart of ART on bulk PBMCs (Figs 2B-D and S1). At the time-point before ART resumption, total and integrated DNA as well as LTR-RNA were significantly higher as compared to baseline ($p<0.0001$, <0.05 and <0.05 respectively). By week 12 post ART resumption, integrated DNA

S2 Table. Adverse events (AE).

All adverse events	n (%)
Any AE	13 (81·3)
Gastrointestinal disorders	3 (18·8)
Abdominal pain	1 (6·3)
Soft faeces	1 (6·3)
Stomatitis	1 (6·3)
Vomiting	1 (6·3)
General disorders and administration site conditions	7 (43·8)
Fatigue	3 (18·8)
Influenza like illness	4 (25·0)
Infections and infestations	8 (50·0)
Bronchitis	1 (6·3)
Ear infection	1 (6·3)
Fungal infection	1 (6·3)
Influenza	1 (6·3)
Nasopharyngitis	4 (25·0)
Oral herpes	1 (6·3)
Pyelonephritis	1 (6·3)
Rhinitis	1 (6·3)
Tracheitis	1 (6·3)
Metabolism and nutrition disorders	1 (6·3)
Vitamin D deficiency	1 (6·3)
Musculoskeletal and connective tissue disorders	3 (18·8)
Arthralgia	1 (6·3)
Back pain	1 (6·3)
Fasciitis	1 (6·3)
Psychiatric disorders	1 (6·3)
Insomnia	1 (6·3)
Renal and urinary disorders	1 (6·3)
Dysuria	1 (6·3)
Respiratory, thoracic and mediastinal disorders	3 (18·8)
Cough	2 (12·5)
Oropharyngeal pain	1 (6·3)
All adverse events related to treatment interruption	
Any AE	3 (18·8)
General disorders and administration site conditions	2 (12·5)
Fatigue	1 (6·3)
Influenza like illness	1 (6·3)
Respiratory, thoracic and mediastinal disorders	1 (6·3)
Oropharyngeal pain	1 (6·3)

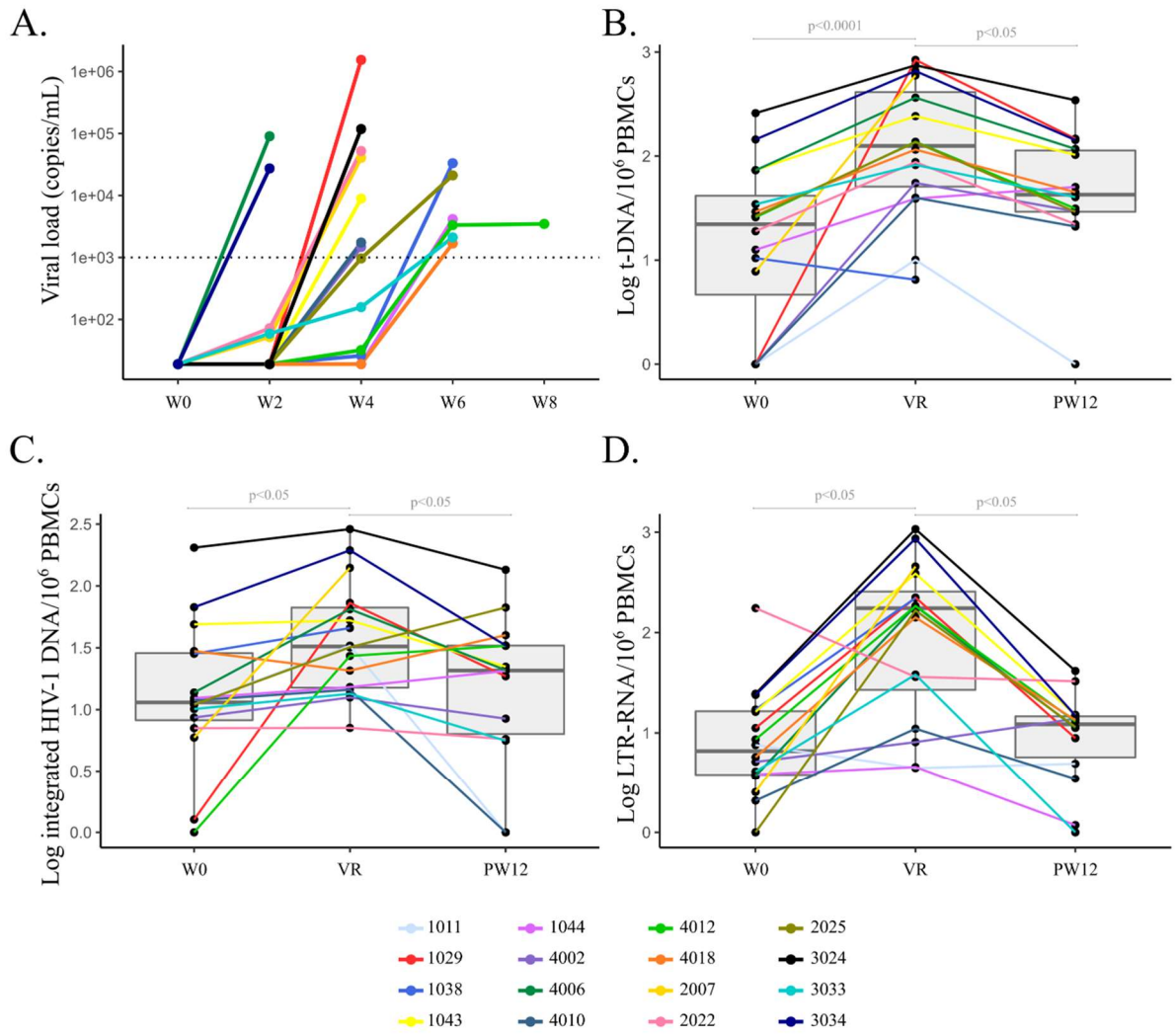
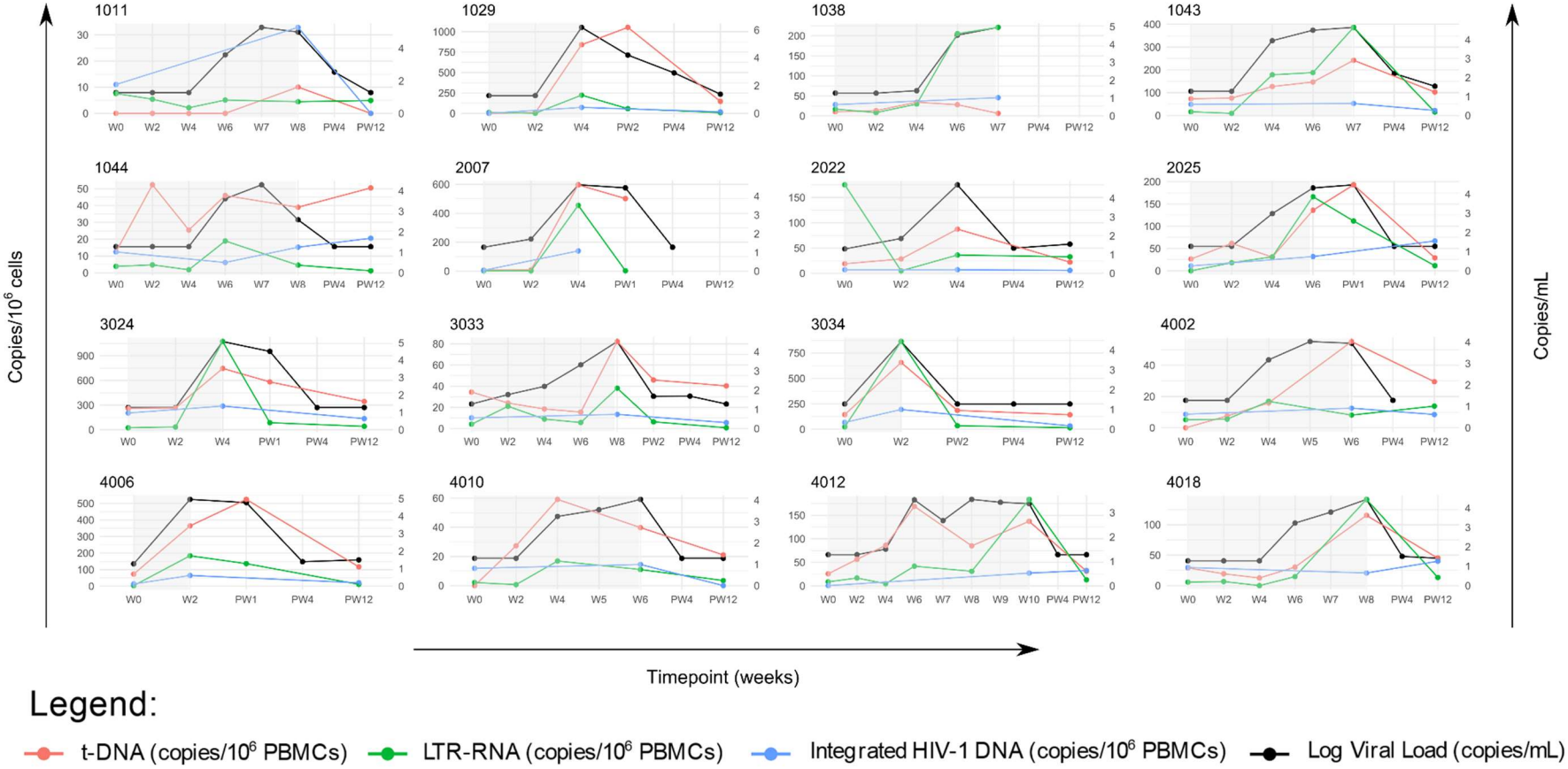


Figure 2. **Evolution of viral parameters after ATI.** (**A**) Plasma viral rebound dynamics after ATI until ART restart in 16 selected patients with very small viral reservoir, (**B-D**) Dynamics of total and integrated HIV-1 DNA and LTR HIV-1 CA-RNA over time in stage 2 patients. P-values are from Friedman Rank Sum tests and post-hoc Nemenyi test. (W: weeks; W0: timepoint at stop ART, VR: timepoint at viral rebound just before restart ART, PW12: 12 weeks after restart ART)

and LTR-RNA normalized to baseline levels, while t-DNA remained slightly but non-significantly higher than baseline ($p>0.05$).

Time to viral rebound is not associated with residual viremia, *in vitro* viral release, qVOA and LTR-RNA levels

Clinical parameters such as HIV risk group, treatment regimen, time on treatment, nadir and current CD4+ T cell count were not associated with TTVR. To further characterize the 16 strictly



S1 Fig. **Detailed viral parameters after ATI.** Individual dynamics of total and integrated HIV-1 DNA, LTR HIV-1 CA-RNA (copies/10^6 cells) and viral load (copies/mL) over time in all stage 2 patients.

selected stage 2 participants, we measured additional viral parameters at baseline, including residual viremia (usVL), *in vitro* viral outgrowth, *in vitro* viral release and LTR-RNA levels in CD4+ T-cells before ATI.

Only one of the selected participants had a VL >5 copies/mL (5.9 copies/mL) while on ART; nine had a detectable VL <5 copies/mL and 5 had undetectable VLs (Fig. 3A). Plasma from one participant (2025) was not available for testing. There was no association between residual viremia and TTVR.

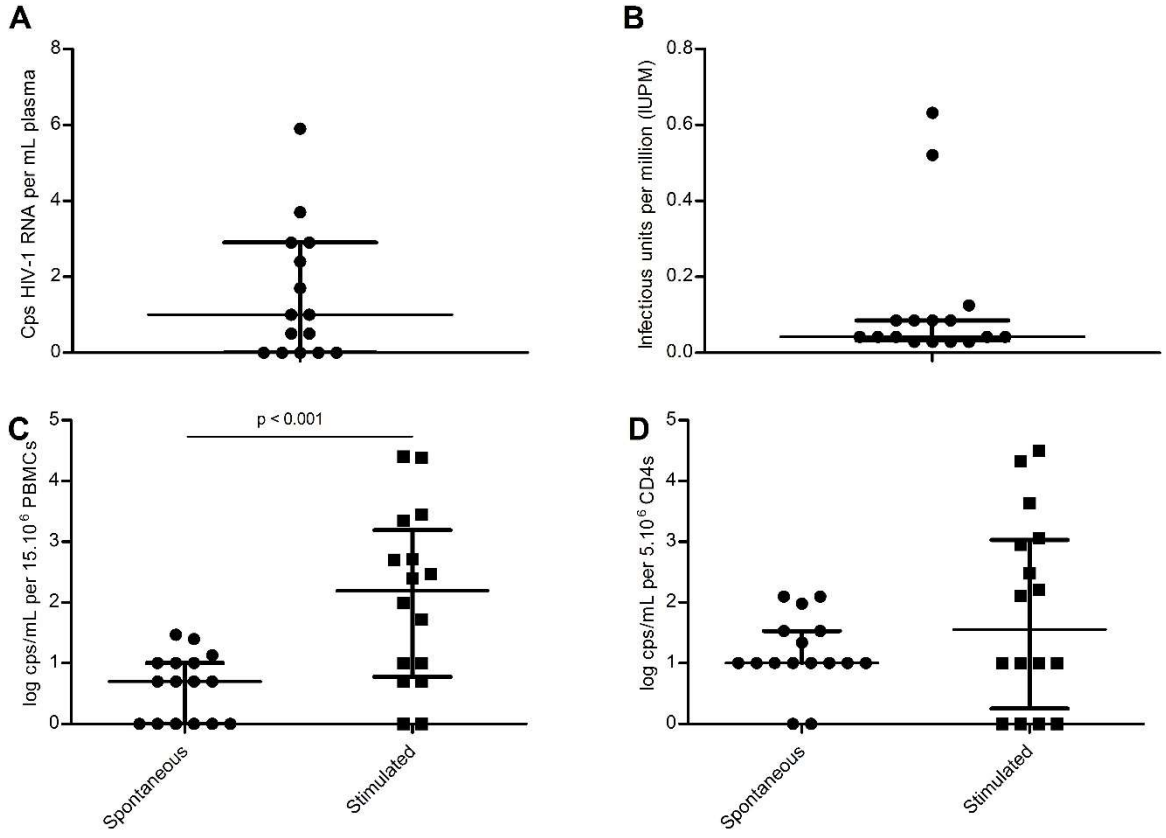
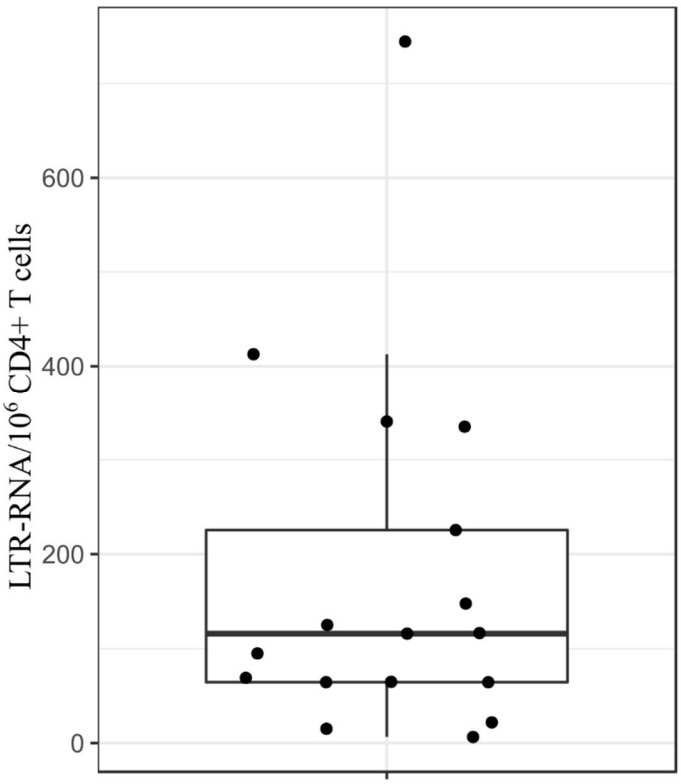


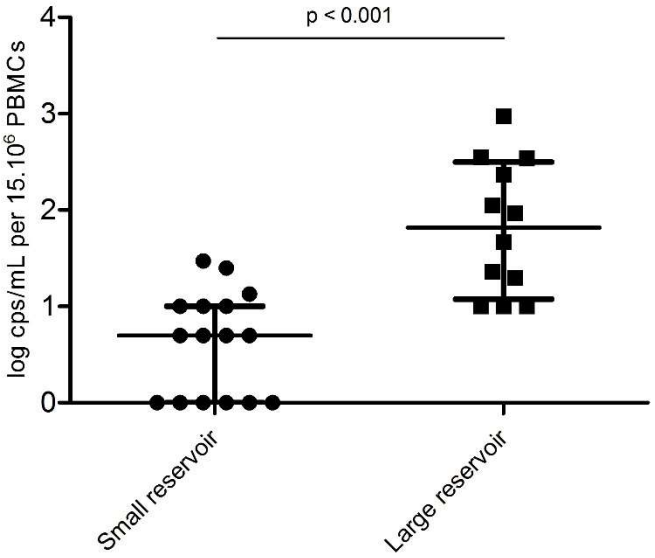
Figure 3. **Additional viral parameters before ATI.** (**A**) Ultra-sensitive plasma viral load (in HIV-1 RNA copies per mL). (**B**) Replication competent fraction (in infectious units per million CD4+ T cells, IUPM).(**C-D**) Viral release from $5x10^6$ PBMCs (C) and CD4+ T cells (D), either spontaneous or after stimulation with PMA + ionomycin (in log copies/mL). P values are from Wilcoxon matched-pairs signed rank test.

In the qVOA assay, very low replication competent fractions were observed in the participants, with a median of 0.042 infectious units per million cells [IQR: 0.032–0.085] (Fig. 3B). Again, there was no association between the size of the replication competent fraction and TTVR. To further distinguish the selected participants based on transcriptional activity, LTR-RNA measurements were repeated at baseline in enriched CD4+ T cells instead of bulk PBMCs. As opposed to US-RNA in PBMCs, LTR-RNA in CD4+ T cells was always detectable and ranged from 6.5 to 744.6 copies per million CD4+ T cells (median: 105.4 copies) but was not associated with TTVR (S2 Fig.).



S2 Figure. Transcriptional activity at baseline. Cell-associated HIV-1 LTR-RNA quantification in CD4+ T cells in stage 2 patients at baseline.

Finally, the recently described VRA was performed. Spontaneous viral release from PBMCs was significantly lower in stage 2 participants as compared to a selection of 12 stage 1 participants with the highest t-DNA levels (median 441 copies per million PBMCs) (S3 Fig.).

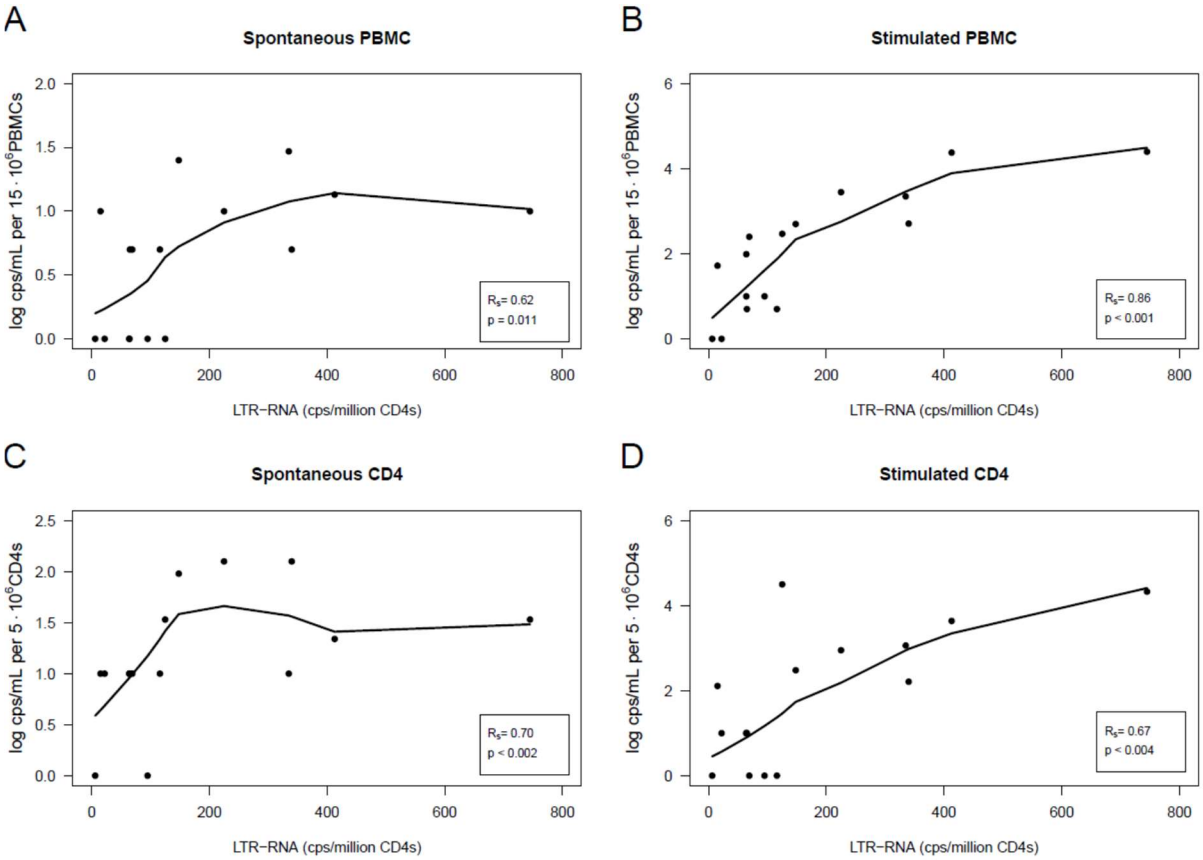


S3 Figure. Viral release. Spontaneous viral release from $15x10^6$ PBMCs before ATI without stimulation in patients with a small and large viral reservoir (in log copies/mL). P-value is from Mann-Whitney test.

Significantly larger amounts of virus were spontaneously released per million CD4+ T cells as compared to PBMCs (median 1.00 versus 0.22 log copies/mL, $p<0.05$) as well as with stimulated versus non-stimulated PBMCs (median 1.77 versus 0.22 log copies/mL, $p<0.001$) (Figs 3C and 3D). Nevertheless, TTVR was not associated with spontaneous or stimulated viral release in PBMCs or CD4+ T cells. Interestingly, at baseline, the level of LTR-RNA in CD4+ T cells significantly correlated with the amount of viral RNA released in the supernatant of PBMC or CD4+ T cells with or without stimulation as measured in the VRA assay (S4 Fig.).

Origin of viral rebound includes circulating CD4 T cells with replication-competent virus in qVOA

To examine the relationship between *in vivo* viral rebound and *in vitro* viral outgrowth viruses, V1-V3 *env* sequences obtained by SGA were compared between plasma rebound viruses and qVOA viruses at baseline in the three participants with more than two positive qVOA wells

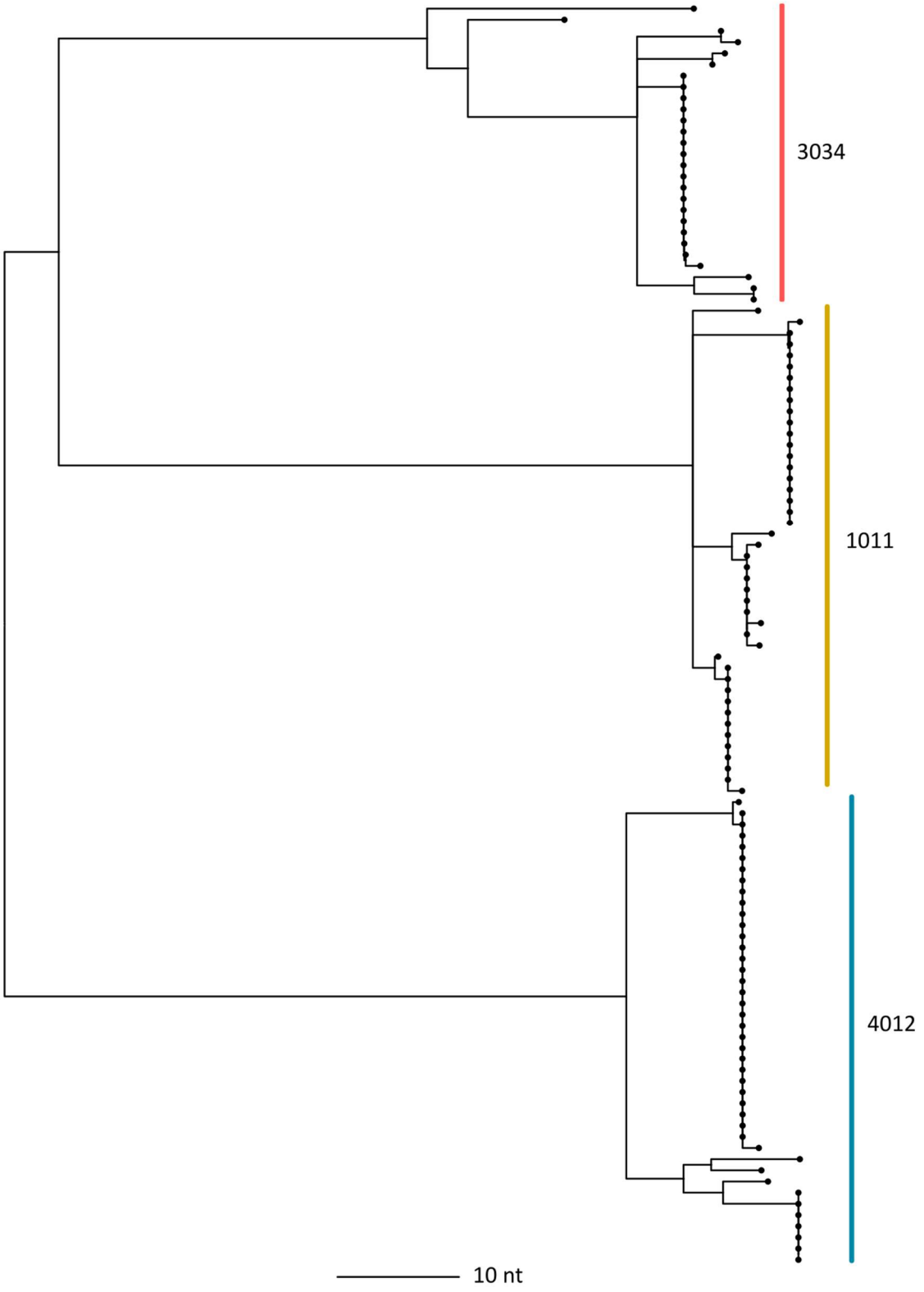


S4 Figure. **Transcriptional activity versus viral release**. Correlation analysis between HIV-1 LTR-RNA and viral release assay. Spearman correlation coefficients were calculated and Locally Weighted Scatterplot Smoothing (LOWESS) curves are depicted.

(1011, 4012 and 3033). A total of 125 plasma rebound sequences were compared to 24 sequences obtained from qVOA. Rebound and qVOA sequences phylogenetically clustered together for each participant (S5 Fig.).

When analysing the individual maximum likelihood phylogenetic tree (Fig. 4), no identical sequences were found between rebound and qVOA viruses for two participants (4012 and 3034), although all sequences intermingled and were phylogenetically closely related. In contrast, in participant 1011, all 12 qVOA viruses were also found in the rebound plasma and clustered together in three distinct but closely related clusters.

To estimate the probability that identical viruses in the V1-V3 *env* region truly represent viral clones, the clonal prediction score described by Laskey *et al*. 2016 was calculated [203]. For the



S5 Figure. Single genome analysis patient clusters. Neighbor-joining phylogenetic tree with all V1-V3 env sequences obtained from participants 3034, 1011 and 4012. Distinct clustering shows no relation between V1-V3 env sequences from different participants.

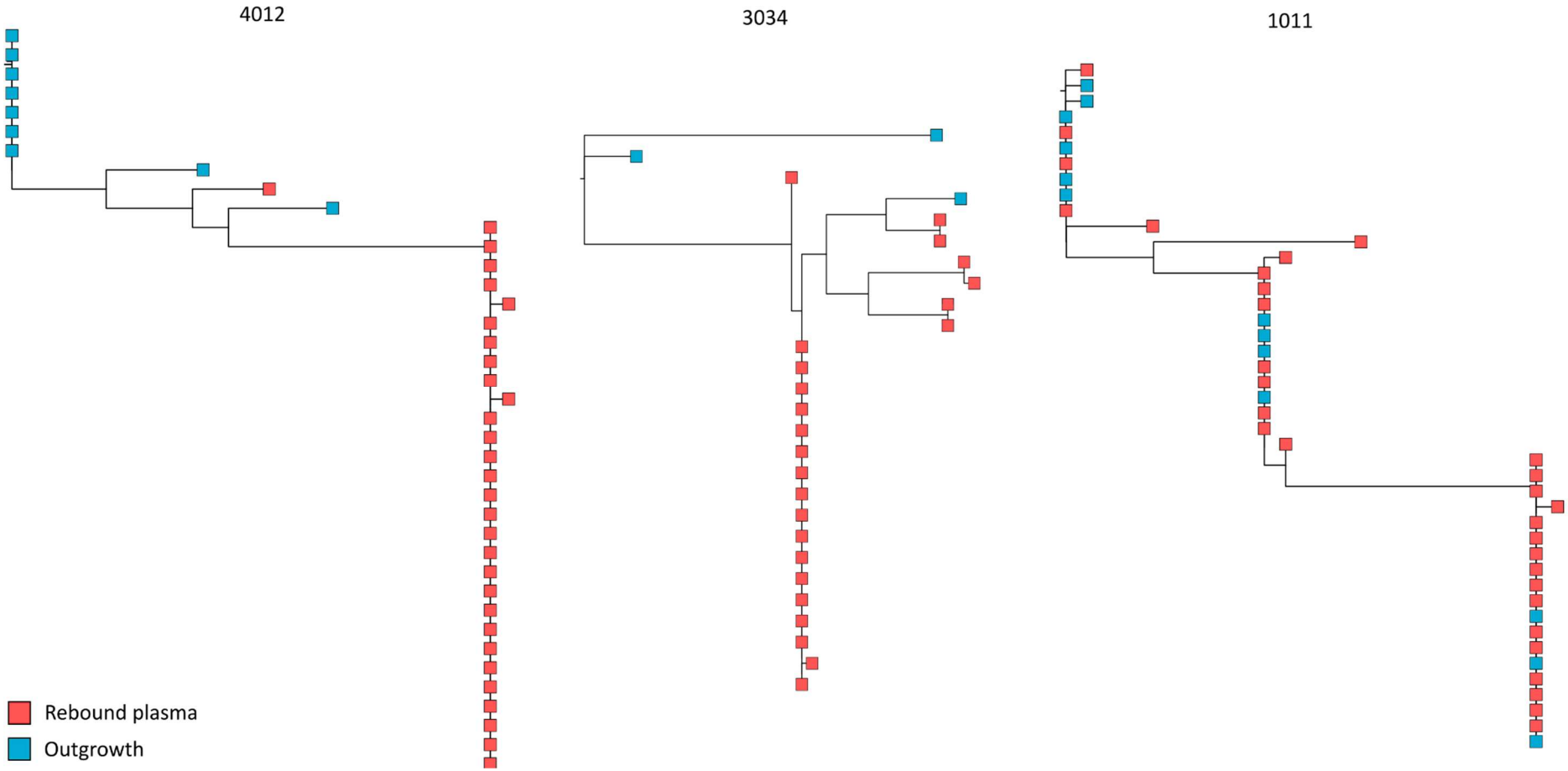


Figure 4. Single genome analysis. Maximum likelihood phylogenetic trees of V1-V3 *env* sequences from rebound plasma and positive qVOA wells (outgrowth) for participants 4012, 3034 and 1011.

V1-V3 *env* region, this score is 88.95 (SD 15.605), giving 89% chance that two or more viruses are identical over the entire genome if they are identical in the V1-V3 *env* region, based on 45 publicly available unique detectable full-genome sequences derived from qVOAs and rebound plasma.

Discussion

In the present study, we hypothesized that ART treated HIV-infected individuals selected for both very low t-DNA and US-RNA, would have a delayed viral rebound after interrupting ART. However, all 16 selected participants rebounded within eight weeks after ATI, which is similar to what has been observed in other ATI studies using less stringent criteria [174, 204]. In line with most ATI studies, however, no major adverse events were recorded and all reservoir measures returned to baseline after treatment restart [186, 204, 205].

It is important to consider that most patients (93%) with below-threshold levels of t-DNA also had below-threshold levels of US-RNA. Consequently, participants undergoing ATI were primarily selected on reservoir size and less so on transcriptional activity. The rapid viral rebound observed in this study is therefore consistent with a recently published ATI study which included patients selected on small reservoir size only, and observed viral rebound in nine out of 10 patients within four weeks (8 patients) and 12 weeks (1 patient) while just one patient maintained plasma viral load below 400 copies/mL for 56 weeks [204].

Exploratory objectives included an association analysis between TTVR and demographic, clinical, and biological parameters. No correlations were observed between these parameters and TTVR. In view of these results, we decided post-hoc to quantify on-going viral transcription by LTR-RNA at baseline as a measure of transcription initiation as opposed to transcription completion (US-RNA). As recently observed by Yukl *et al* [197], LTR-RNA levels were much higher

than US-RNA levels (S2 Fig.). Interestingly, LTR-RNA levels correlated significantly with the amount of virus released in *in vitro* culture (VRA) but were not associated to TTVR. Future ATI studies could consider using such markers of early transcription to select potential participants, as they are more sensitive than US-RNA.

To investigate the origin of viral rebound, we compared qVOA outgrowth viruses with *in vivo* plasma rebound viruses using SGA in three participants with more than two positive qVOA wells. Exact matches between qVOA and rebound clones have been reported only very rarely [206, 207]. In these three participants, we observe intermingling between the viral sequences obtained from qVOA and plasma at viral rebound, confirming their close phylogenetic relatedness. Interestingly, in one participant, three distinct viral sequence expansions from the qVOA had exact matches with sequence expansions observed in the V1-V3 *env* rebound sequences. These data suggest that qVOA sequence data might in some cases predict the origin of viral rebound. Nevertheless, the comparison was limited to the highly variable but relatively small V1-V3 sub genomic region of Env. The clonal prediction score of this amplicon is 89%, leaving an 11% chance that the matching viruses differ in one or more nucleotides somewhere else in the genome. However, this prediction score was calculated based on a limited database of 45 unique detectable sequences and therefore has a high standard deviation, making it difficult to interpret this score.

This study had a number of limitations. First, a limited number of participants was included with heterogeneous baseline characteristics (Table 1). Second, all participants experienced viral rebound within a relatively short time frame, making differences in TTVR very small (0-6 weeks) and correlations unlikely, considering the small sample size. Third, participants were monitored only every other week. More frequent sampling (e.g. twice weekly) could have

allowed a more refined analysis of rebound kinetics and more powerful correlation analyses. A fourth limitation, inherent to all recent HIV ATI studies, is that ART was generally restarted immediately or shortly after meeting the stringent viral rebound criteria. This may have prevented the detection of viral control after a transient viral rebound, as is observed in a large subset of PTCs as late as 24 weeks after ATI [187]. Indeed, one participant experienced viral rebound after eight weeks but maintained pVLs below 3,500 copies/mL for 11 weeks, after which the participant decided to restart therapy. Considering the good safety observed in recent ATI studies [200], and the lower probability of missing PTCs [187], future trials might consider adopting higher treatment restart thresholds. These advantages have to be weighed against the risk of exposing participants and their partners to longer periods of detectable viral loads and subsequent risk of transmission [208]. Finally, we were limited to peripheral blood parameters, while the largest proportion of the HIV reservoir is found in various tissues throughout the body, which are much more difficult to sample exhaustively [58].

In conclusion, although HIV-1 reservoir markers such as t-DNA and US-RNA have been correlated with PTC status, we show here that their predictive power is too weak to prospectively identify PTCs from the general patient population on ART. PTC status most likely depends not only on the size, transcriptional activity and replication competence of the viral reservoir in the peripheral blood, but also on reservoirs elsewhere and the immune activity in the body. Developing reliable and easy assays to evaluate whole body parameters of viral activity and immune control remains a major challenge for HIV cure research.

Supplementary methods

S1 Methods. Cell-associated HIV-1 DNA and RNA measurements at screening

t-DNA and US-RNA targeting the gag/pol region were measured at screening by droplet digital PCR (ddPCR) as previously described [194]. Briefly, genomic DNA (gDNA) was extracted from 10 million PBMCs by the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) using manufacturer's protocol with an additional step of heating 75µl elution buffer on the column at 56°C for 10min. Before amplification, 8.65µl of genomic DNA was restricted by EcoRI in a total volume of 10µl restriction digest. RNA was extracted from a separate vial of 10 million PBMCs by Innuprep RNA kit in 30µl elution buffer (Westburg, Leusden, The Netherlands). Extraction yields were verified by Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Geel, Belgium). A total of 1,000ng RNA was reverse transcribed to cDNA by qScript cDNA SuperMix (Quantabio, Beverly, MA, USA) and used for further quantification.

t-DNA and US-RNA were measured by ddPCR by adding respectively 2µl and 4µl in triplicates to ddPCR mix containing 10µl 2x ddPCR Supermix for Probes, respectively 800nM/500nM primers and 300 nM probe (Integrated DNA Technologies (IDT), Belgium). Two sets of primers and probe were used for US-RNA depending on the HIV-1 subtype to ensure adequate pick-up of HIV-1 non-B subtypes (primers and probes are depicted in S2 Table). PCR amplification was performed with an initial denaturation step of 5min at 95°C followed by 40 cycles of a denaturation step for 30sec at 95°C and annealing/elongation for 1min at 58°C. Droplets were read by QX200 droplet reader (Bio-Rad) and analyzed using ddpcRquant software [209].

t-DNA was normalized by measuring the reference gene RPP30 in duplicate by ddPCR and expressed per million PBMCs. US-RNA was normalized by dividing copies HIV RNA by the geometric mean of three reference genes per patient, B2M, YMHAZ and HMBS, measured

with LightCycler480 SYBR Green I Master mix (Roche Applied Science, Belgium) and expressed per million cells by normalizing to the theoretical number of cells per µl RNA.

Cell-associated HIV-1 DNA and RNA measurements during stage 2

In stage 2 participants, t-DNA and cell-associated LTR HIV-1 RNA (LTR-RNA) were measured in PBMCs at baseline, on weeks 2, 4, 6, 8, 12, 16, 20, 24, 32, 40 and 48 post-ATI and week 12 post-ART restart (PW12) (Fig. 1A), integrated HIV-1 DNA at baseline, before restart ART and PW12, and LTR-RNA in CD4+ T cells at baseline. LTR-RNA measures not only the unspliced HIV-1 RNA but targets all HIV transcripts elongated beyond 5'-LTR region [191, 197]. For this particular assay, RNA was reverse transcribed to cDNA by qScript XLT cDNA SuperMix (Quantabio, MA, USA) allowing a maximum input of 2,000ng instead of 1,000ng. To increase the sensitivity of LTR-RNA quantification at baseline a second measurement was performed on enriched CD4+ T cells: CD4+ T cells were isolated from PBMCs at baseline using EasySep Human CD4+ T Cell Isolation Kit (Stemcell technologies, Vancouver, Canada). ddPCR for HIV DNA and HIV RNA quantification was performed as described above.

Integrated HIV-1 DNA was measured by repetitive sampling of Alu-HIV PCR based on Poisson statistics as described previously [210]. Briefly, 40 replicates were analyzed by a nested qPCR with a first round targeting human Alu gene and HIV-1 LTR, followed by a second round targeting HIV LTR (primers and probe in S2 Table). To correct for background noise, 20 replicates without the forward Alu primer were run in parallel. The input of genomic DNA was set at 2 copies of total HIV-1 DNA per replicate. The number of copies integrated HIV-1 DNA was calculated based on the number of positive wells with Poisson statistics and normalized per million PBMCs by reference gene RPP30 quantification by ddPCR.

S2 Methods. Quantitative viral outgrowth assay (qVOA)

This assay was based on Laird et al. with some modifications [193]. In short, 24 x 10^6 CD4+ T cells, enriched from PBMCs using positive selection (Miltenyi, Bergisch Gladbach, Germany), were plated on day 0 in two 24 well plates at 5 x 10^5 cells/well together with 5 x 10^6 γ-irradiated allogeneic PBMCs from uninfected donors in 2mL RPMI (Westburg, Leusden, The Netherlands) containing 15% FCS (Life Technologies, Ghent, Belgium), 200U/mL interleukin-2 (IL-2, Gentaur, Kampenhout, Belgium) and 1µg/mL phytohaemagglutinin PHA (Thermo Scientific, Merelbeke, Belgium). Donor PBMCs were isolated from buffy coats, obtained from the Red Cross Transfusion Center in Mechelen, Belgium. On day 1, the cells were washed twice and 10^5 MOLT-4/CCR5 cells (NIH AIDS Reagent Program) were added per well. IL-2 containing medium was refreshed twice per week and supernatant of each well was tested for infectious HIV-1 after one, two, three and four weeks of culture with the TZM-bl assay (NIH AIDS Reagent Program) [211]. The frequency of replication-competent latently infected cells was calculated by a maximum likelihood method, as described previously, and is expressed as infectious units per million cells (IUPM) [40].

Chapter 5 - *In vitro* viral suppressive capacity correlates with immune checkpoint marker expression on peripheral CD8+ T cells in treated HIV positive patients

Author's contributions: The following chapter is entirely the author's work, except for the phenotypic analyses by flow cytometry which were performed by the second first author of the paper, Philipp Adams from the Luxemburg Institute of Health.

In-vitro viral suppressive capacity correlates with immune checkpoint marker expression on peripheral CD8+ T cells in treated HIV positive patients

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Abstract

Objective: To determine whether viral suppressive capacity (VSC) of CD8+ T cells can be boosted by stimulation with HIV-1 peptides and whether the ability to control HIV-1 replication correlates with immunological (cytokine production and CD8+ T cell phenotype) and viral reservoir measures (total HIV-1 DNA and cell-associated RNA) in well-treated HIV-infected chronic progressors.

Design: We compared VSC of peripheral CD8+ T cells to cytokine production profile in response to peptide stimulation, detailed phenotype (17-color flow-cytometry), reservoir size (total HIV-1 DNA), basal viral transcription (unspliced cell-associated RNA) and inducible viral transcription (*tat/rev* induced limiting dilution assay) in 36 HIV+ patients on cART and six healthy donors.

Results: We found that the VSC of CD8+ T cells can be increased by prior stimulation with a pool of consensus HIV-1 *gag* peptides in a significant proportion of progressor patients. We also found that VSC after peptide stimulation was correlated with higher expression of immune checkpoint markers on subsets of terminally differentiated effector memory (TEMRA) CD8+ T cells as well as with production of IFN-γ, TNF-α and IL-10. We did not find a correlation between VSC and viral reservoir measures.

Conclusions: These results add to a small body of evidence that the capacity of CD8+ T cells to suppress viral replication is increased after stimulation with HIV-1 peptides. Interestingly, this VSC was correlated with expression of immune checkpoint markers, which are generally considered to be markers of exhaustion. Our findings may guide further investigations into immune phenotypes correlated with viral suppression.

Introduction

HIV-1 persists in a reservoir of long-lived latently infected cells [39, 212]. Most HIV-1 infected subjects are dependent on combination anti-retroviral therapy (cART) to suppress viremia [173, 213] ('progressors'), while a small proportion controls viremia without treatment ('elite controllers') or after a period of cART ('post-treatment controllers') [54]. The concept of "functional cure" or "viral remission" is to induce viral control without cART in 'progressors' [214-217].

It is generally accepted that a sufficiently reduced viral reservoir and/or potent cytotoxic T cell (CTL) responses are needed to achieve viral remission [218]. Indeed, lower levels of HIV DNA and cell-associated RNA in peripheral blood mononuclear cells (PBMCs) before structured treatment interruption in chronically infected subjects have been associated with delayed time to viral rebound [181, 219]. Studies comparing the direct ex-vivo CTL potential of elite controllers and classical progressors illustrate the importance of polyfunctionality and viral suppressive capacity (VSC) of CD8+ T cells in the control of viremia [112, 134, 136]. Furthermore, a correlation between lower pre-therapy expression of immune checkpoint markers on T cells and delayed viral rebound after treatment interruption was shown in the SPARTAC trial [220].

To reduce the size of the viral reservoir, the shock-and-kill strategy, using latency reversing agents, is one currently attempted approach [221]. On the other hand, inducing potent CTL responses might be necessary and could be achieved by therapeutic vaccination. In this respect, in-vitro models evaluating the VSC of CD8+ T cells could help in the evaluation of vaccine candidates before going into clinical trials.

Viral inhibition assays generally use CD8+ T cells which are either unstimulated or stimulated in a polyclonal, non-specific way. According to most published data, this type of VSC is absent

or very low in 'progressors', even if they are treated with cART [112, 134, 135]. There are some indications that VSC can be induced by HIV-specific stimulation in-vitro [107, 110, 222], but whether this is the case in all or only some progressors and whether or how this immune function correlates with other immune and viral markers, has not been studied in-depth until now.

Because *gag*-specific immune responses have been associated with protective immunity [114, 223-225], we set up a viral inhibition assay in which patient-derived CD8+ T cells are specifically stimulated with a pool of HIV-1 *gag* peptides before co-culturing them with super-infected autologous CD4+ T cells. We hypothesized that variation in *gag*-stimulated VSC exerted by CD8+ T cells from well-treated progressors will be observed and that it will correlate with some immunological parameters, such as cytokine production levels or CD8+ T phenotypes, or with viral reservoir measures, such as total HIV DNA and levels of basal and induced viral transcription in CD4+ T cells. Investigating this conceptually important anti-viral function of CD8+ T cells in well-treated progressors and studying its correlation with established immune and viral parameters will provide useful "baseline" knowledge for future immune-based interventions in this large patient population that is to be included in future interventions towards "remission" or "cure".

Methods

Study subjects and samples

Thirty-six chronic HIV-1 infected (progressor phenotype), asymptomatic, cART-treated, adult patients were recruited at the Institute of Tropical Medicine (ITM) in Antwerp. Inclusion and exclusion criteria are given in supplementary methods. One hundred and fifty mL of EDTA blood was collected from these patients. We also obtained buffy coats from six healthy HIV-seronegative adult controls from the Red Cross Blood Transfusion Center of Mechelen,

Belgium. All participants gave written informed consent per the Declaration of Helsinki under Antwerp ITM Review Board-approved protocols (Belgian registration number: B300201526243).

PBMCs were isolated by Ficoll-Hypaque (Axis-Shield, Oslo, Norway) density gradient centrifugation, resuspended in 90% FBS 10% DMSO, kept overnight in Mr. Frosty boxes (Thermo Fischer Scientific, Waltham, MA, USA) at -80°C and stored in liquid nitrogen until use.

Viral inhibition assay

Viral suppressive capacity (VSC) was measured using a viral inhibition assay that was based on a published protocol with significant modifications [222].

For the preparation of CD4+ T target cells, PBMCs were stimulated during seven days at 37°C 7% CO_2 in RPMI 2.5% human serum (HS, A&E Scientific, Belgium), IL-2 (500IU/mL, Gentaur, Kampenhout, Belgium) and anti-human CD3/8 bi-specific monoclonal antibody (1µg/mL, NIH AIDS Reagent Program). For the preparation of stimulated CD8+ T effector cells, PBMCs were incubated during 7 days at 37°C 7% CO_2 in RPMI 2.5% HS and 10µg/mL (81ng/mL per peptide) of an HIV consensus subtype B *gag* peptide pool (NIH AIDS Reagent Program). For the preparation of non-stimulated CD8+ T effector cells, an extra aliquot of PBMCs was thawed one day before the start of CD4+/CD8+ T cell co-culture and rested overnight at 37°C 7% CO_2 in RPMI 2.5% HS.

On day 0, CD4+ T target cells were enriched by negative selection magnetic beads (Miltenyi Biotech, San Diego, CA, USA) from the anti-CD3/8 mAb stimulated PBMCs. Stimulated as well as non-stimulated overnight rested CD8+ T effector cells were enriched from the *gag* peptide pool stimulated PBMCs and overnight rested PBMCs respectively, by negative selection. Cell purities of enriched CD4+ T and CD8+ T cells >90% were confirmed by staining for CD3 (FITC,

Clone OKT3), CD4 (PE, clone SK3), CD8a (APC-eFluor, clone SK1) and reading on a BD FACSVerse (Beckton Dickinson, New Jersey, USA).

Enriched CD4+ T cells were incubated with HIV III_B (NIH AIDS Reagent Program) at a multiplicity of infection of 0.001 for 3 hours at 37°C 7% CO_2. Infected target cells were washed three times and resuspended at 10^6 cells/mL in RPMI 2.5% HS 500IU/mL IL-2 and cultured in triplicate in flat bottom 96-well plates (VWR, Leuven, Belgium) at 10^5 cells/well, alone (positive control) or in co-culture with stimulated or rested enriched CD8+ effector T cells at an effector-to-target ratio of 1:1 and 2:1. Culture medium was refreshed at days 2, 6 and 9. The level of HIV-1 p24 antigen in the supernatant was determined at day 13 by in-house p24 ELISA [226]. Log inhibition values were calculated on day 13 as $\log_{10}$(p24 without CD8+ T cells) – $\log_{10}$(p24 with CD8+ T cells).

Quantification of HIV-1 DNA and RNA

Total genomic DNA and cell-associated RNA were isolated from 5 x 10^6 PBMCs each. Total HIV-1 DNA and cell-associated HIV-1 unspliced RNA were measured with the QX200 Droplet Digital PCR platform (Bio-Rad, Hercules, CA, USA) as described previously [227, 228].

Total HIV-1 DNA amounts were normalized to copies per 10^6 PBMCs with the reference gene *RPP30*. For absolute HIV-1 usRNA quantification, normalization of input cDNA was done by quantifying gene expression levels of stably expressed reference genes as described previously [229, 230]. Data analysis was performed with ddpcRquant [209].

Tat/rev induced limiting dilution assay

The *tat/rev* induced limiting dilution assay (TILDA) was performed as described previously by Procopio *et al.* [231].

Cytokine production after peptide stimulation

Supernatant of PBMCs stimulated for seven days with the HIV *gag* peptide pool (81ng/mL per peptide) was tested for type 1 interferon (IFN), T helper (Th)1, Th2 and Th17, including IFN-α, IFN-β, IFN-γ, tumor necrosis factor (TNF)-α as well as the following interleukins (IL): IL-4, IL-5, IL-10, IL-17, IL-22 and IL-27 with Luminex technology on a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA, USA). All samples were tested undiluted.

Flow-cytometric phenotyping

Phenotyping of CD8+ T cells at baseline and during co-culture was done with two panels. The "cytotoxicity/activation panel" focused on cytotoxicity and activation markers as well as intra-cellular cytokines and included the following markers: CD3, CD4, CD8, CD107a, IL2, CD56, IFN-γ, Granzyme B, CD45RA, HLA-DR, CD38, CCR7, Perforin, CD57, Zombie NIR fixable viability kit. The "immune checkpoint panel" focused on immune checkpoint markers and transcription factors and included the following markers: CD3, CD4, CD8, CD28, CD27, TIM-3, EOMES, CD160, LAG-3, PD1, 2B4, CD45RA, Tbet, CCR7, TIGIT, Zombie NIR fixable viability kit. A detailed list of all used human species reactive antibodies included in the panels above can be found in the supplementary methods.

Staining of PBMCs for flow cytometry was performed using the FOXP3 transcription factor staining buffer set (eBioscience). Briefly, cells were harvested and washed twice in cold PBS 1% FBS. Next, surface stains and near infrared live dead dye were incubated for 30 minutes. Cells were washed twice, fixed and permeabilized with the eBioscience FOXP3 kit. Cells were then stained intracellularly for 30 minutes and washed twice. Samples were acquired on the Fortessa LSR flow cytometer (BD Immunocytometry Systems, San Jose, CA). Data analysis was performed using FlowJo version 10 and Kaluza version 1.5.

Statistical Analysis

Mann-Whitney U tests were used to compare differences in mean VSC, cytokine levels and phenotypic markers between suppressors and non-suppressors. Linear regressions and Spearman rank tests were used to determine correlations between clinical, virologic and immunological parameters. Family-wise corrections of p-values were made using the Bonferroni-Holm's method.

Cluster analysis of flow cytometry phenotypic data

Flow cytometry data was cleaned and compensated with Flow Jo version 10 software (BD, New Jersey, USA). CD8+ T cell populations were concatenated containing the file internal compensation matrix and reimported into the Cytobank environment. Scaling of channels was applied based on single compensation controls for each marker. Cluster analysis was done by running a SPADE (**S**panning tree **p**rogression **a**nalysis of **d**ensity-normalized **e**vents) algorithm on the mother population of total CD8+ T cells. Clustering was based on CD45RA and CCR7 expression.

SPADE results were verified by manual gating. Median fluorescence intensities (MFI) for each cluster were analyzed in GraphPad Prism and fold changes between suppressors and non-suppressors were calculated as ((MFI(Suppressor) – MFI(Non-suppressor)) / MFI(Suppressor). Multiple t-tests were run to statistically compare all markers between both groups. P-values depicted on figures are not corrected for multiple testing.

Results

Participant Demographics

The demographics of the 36 HIV+ patients recruited in this study are given in Table 1. Patients with a wide range of age, time on therapy, peak viral load and CD4 counts were included in

Table 1. Patient demographics of 36 included HIV+ patients.

Age (years)		CD4 T cell nadir (cells/µL)	
- Range	31.1 – 50.1	- Range	1 – 610
- Median	40.0	- Median	278
Time on cART (months)		Actual CD4 T cell count (cells/µL)	
- Range	18 – 248	- Range	411 – 1504
- Median	86	- Median	664
Peak Viral Load (cps/mL)		Gender	83% male
- Range	5,290 – 1,020,000	Subtype B infected	64%
- Median	244,604	Non-subtype B infected	36%

order to capture the diversity of our clinical cohort. Thirty-one patients had an undetectable HIV viral load (VL), four patients had a detectable VL <50 copies/mL, one patient had a VL of 52 copies/mL.

CD8+ T cell viral suppressive capacity is strongly enhanced after an HIV specific in-vitro peptide stimulation in a subset of progressor patients

We measured VSC of non-stimulated and subtype B *gag* peptide-stimulated CD8+ T cells in six healthy HIV negative volunteers and in 36 HIV-1 infected individuals at effector to target ratios (E:T) of 1:1 and 2:1. Our assay proved to be specific for HIV-1 as minimal suppression was observed in all healthy volunteers, with or without peptide stimulation, whereas strong suppression was observed in some HIV-1 infected individuals (Fig. 1A). Based on the comparison with healthy controls, “suppressors” were defined as having a VSC with peptide stimulation exceeding the average VSC of healthy volunteers + three standard deviations, corresponding to 0.34 $\log_{10}$.

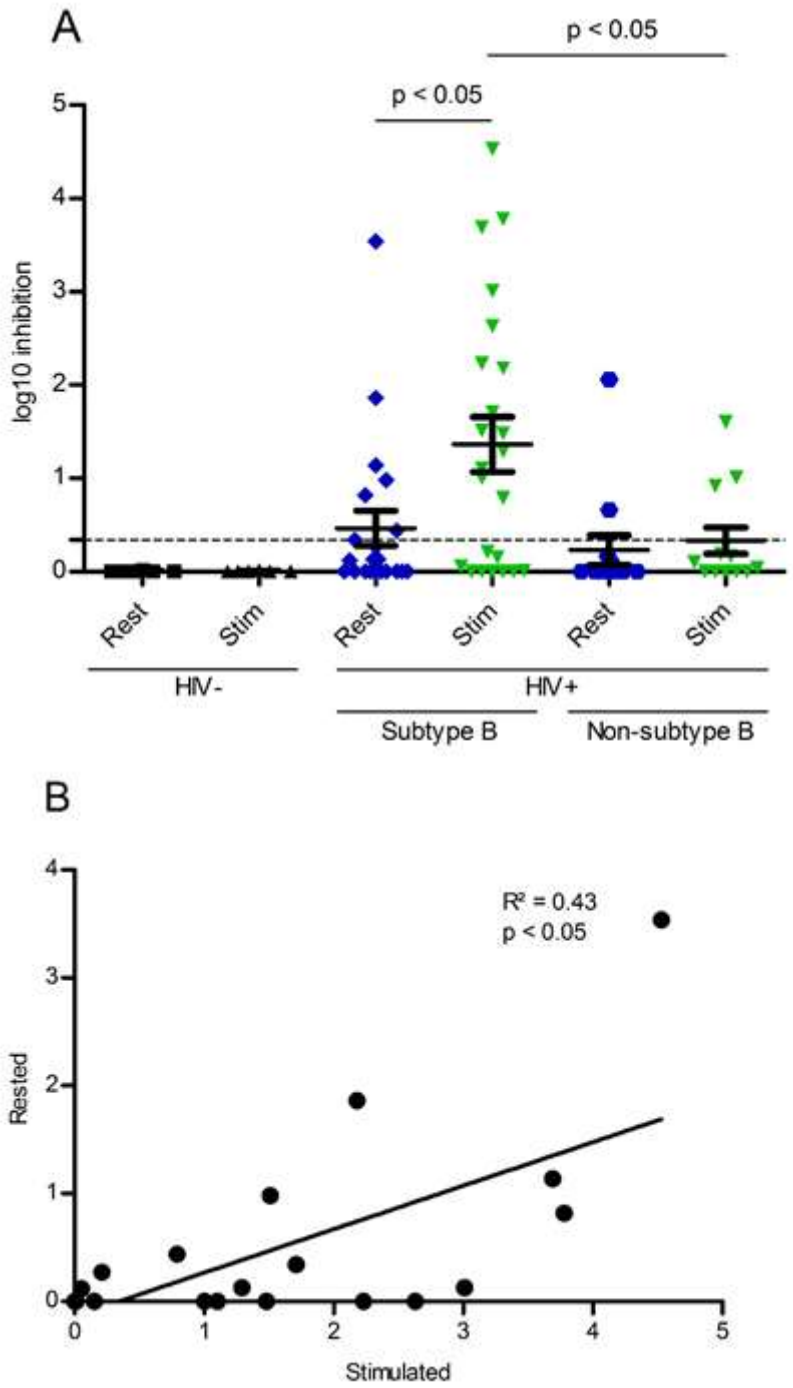


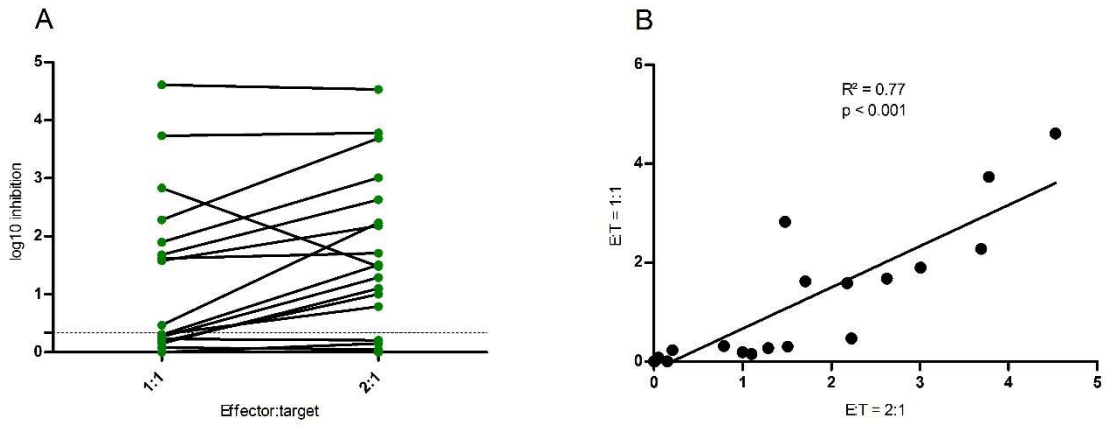
Figure 1. Viral suppressive capacity. **(A)** Log_{10} inhibition values of HIV- people and HIV+ patients (infected with subtype B and non-subtype B HIV-1 strains) with overnight rested (Rest) and 7-day peptide stimulated (Stim) CD8+ T cells as measured in the viral inhibition assay with an E:T of 2:1. Mean with SEM is depicted. The dashed line represents the threshold of 0.36log above which patients are defined as suppressors. **(B)** Correlation between viral suppressive capacity with rested versus 7-day peptide stimulated CD8+ T-cells with an E:T of 2:1.

In subtype B infected individuals, 6 out of 23 (26%) suppressed viral replication without peptide stimulation (mean: 0.47 log_{10}) whereas 14 out of 23 (61%) suppressed viral replication with peptide stimulation (mean: 1.36 log_{10}). The difference in mean response was significant ($p < 0.05$). As expected, no increase in suppression was observed in patients infected with non-subtype B HIV strains after stimulation with the consensus B peptide pool, presumably because these patients have no memory T cells specific for subtype B antigens (Fig. 1A).

Furthermore, we observed a strong correlation ($p < 0.05$) in subtype B infected patients between VSC with and without peptide stimulation (Fig. 1B). As expected, inhibition values were higher at an E:T of 2:1 as compared to 1:1 for most patients (Suppl. Fig. 1A). VSC at both ratios strongly correlated with each other (Suppl. Fig. 1B). On the other hand, VSC did not correlate with current and nadir CD4+ T cell count or peak VL. Nevertheless, in subtype B infected patients VSC with and without peptide stimulation is on average higher in patients with detectable plasma VL as compared to patients with an undetectable VL (1.68 log versus 1.30 log and 0.89 log versus 0.36 log respectively), but these differences are not significant.

HIV viral reservoir measures do not correlate with viral suppressive capacity

Total HIV-1 DNA and usRNA were measured in all 23 subtype B infected patients. HIV-1 DNA was detectable in all patients and ranged from 15 to 859 copies per million PBMCs (mean: 238) (Fig. 2A). Unspliced HIV-1 RNA was detected in 17 out of 23 patients (74%) and ranged from 0.0 to 32.9 copies per million PBMCs (mean: 6.7) (Fig. 2B). These values are consistent with the literature on reservoir size of individuals on cART with suppressed viremia. Total HIV-1 DNA and usRNA did not correlate with VSC or any other studied clinical or immunological parameters.



Supplementary figure 1. (A) Log_{10} inhibition values of HIV+ patients (infected with subtype B HIV-1 strains) as measured in the viral inhibition assay with an E:T of 1:1 and 2:1. **(B)** Linear regression analysis of VSC at E:T of 1:1 versus 2:1.

To further characterize the "functional" viral reservoir, we performed the TILDA assay on 21 available subtype B HIV-1 infected patients. Without PMA/Ionomycin stimulation, no significant transcriptional activity of *tat/*rev RNA was observed (Fig. 2C). With stimulation however, six had detectable amounts of transcription, with values ranging from 55.8 to 313 cells with detectable HIV RNA transcripts per million CD4+ T cells. Therefore pro-viral transcription was inducible with PMA/ionomycin in only a minority (6/21 = 29%) of the patients. VSC did not correlate with induced viral transcription. TILDA inversely correlated with CD4 nadir ($R = -0.67$; $p < 0.05$).

Cytokine Production in Response to Peptide Stimulation

Next, we investigated whether cytokine production in response to HIV peptide stimulation could be predictive of VSC. To this end, we determined cytokine levels in supernatant from PBMCs stimulated for seven days with the HIV-1 *gag* peptide pool, before the start of the CD4+/CD8+ T cell co-culture in 26 available patient samples.

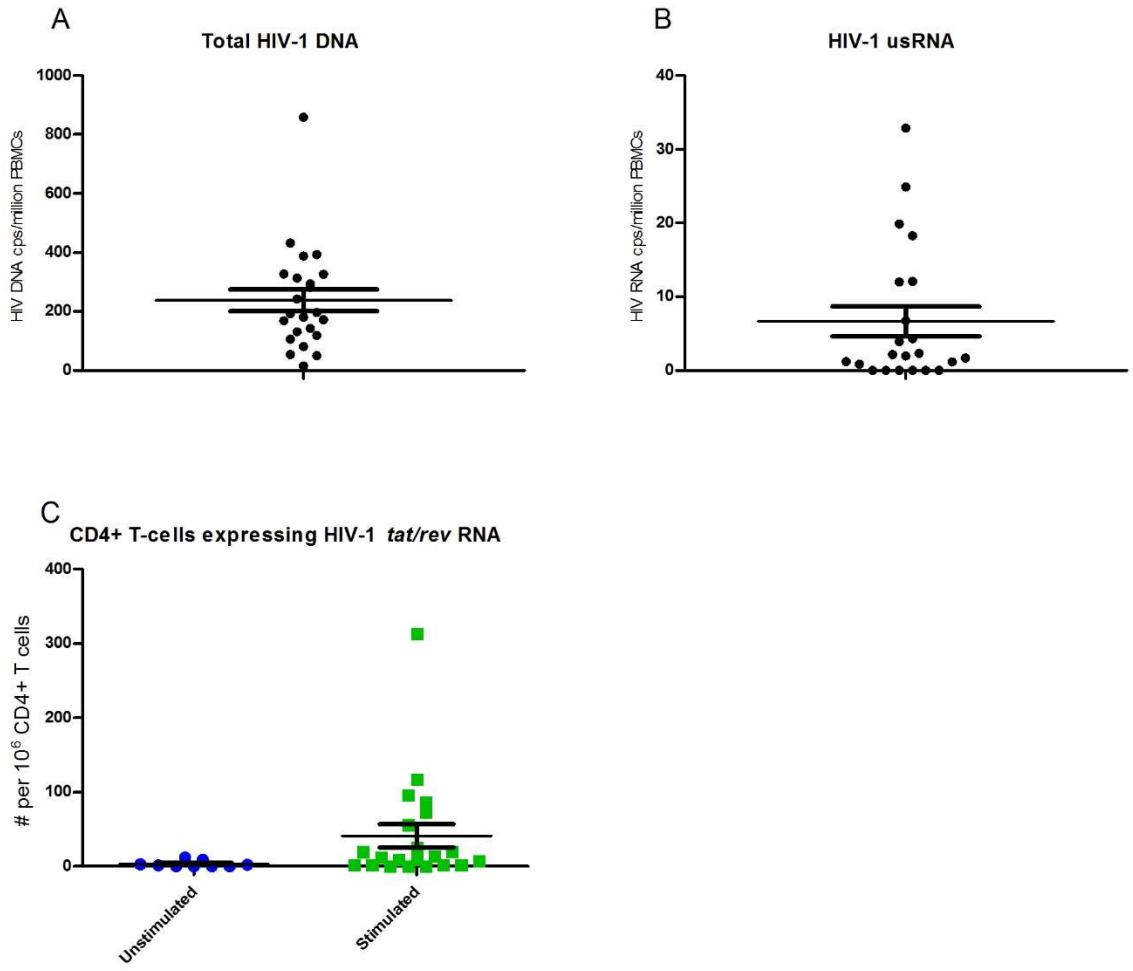


Figure 2. Viral reservoir measures. (A) Total amount of HIV-1 DNA and (B) cell-associated unspliced RNA copies per million PBMCs for all 23 HIV+ subtype B infected patients. Mean with SEM is depicted. (C) Tat/rev induced limiting dilution assay (TILDA) results in number of cells with detectable multiply spliced HIV-1 mRNA per million CD4+ T cells without (N = 10) and with PMA/ionomycin stimulation (N = 21).

When comparing mean cytokine levels, only IFN-γ was produced significantly more in suppressors than in non-suppressors ($p < 0.01$) (Fig. 3A). While TNF-α and IL-10 levels did not differ significantly, their ratio was significantly higher in suppressors than in non-suppressors ($p < 0.05$) (Fig. 3B). Together, these results point towards a correlation between Th1 responses and VSC.

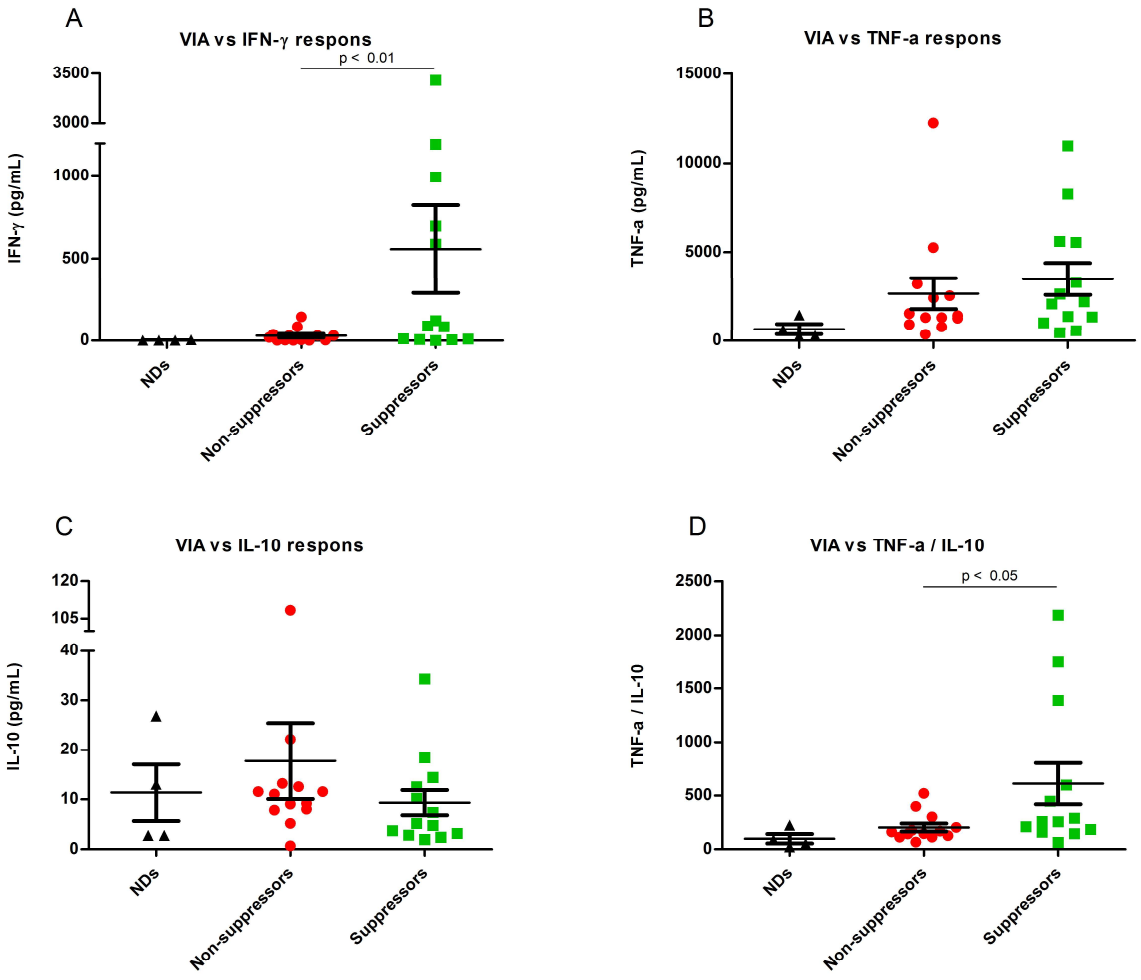


Figure 3. Cytokine production. Cytokine levels in supernatant of PBMCs after 7 days of stimulation with a consensus subtype B HIV-1 *gag* peptide pool from HIV-infected progressors with either a subsequent "suppressor" or "non-suppressor" activity in CD4+/CD8+ co-culture, as compared to HIV-negative subjects (HIV negative donors or ND). (VIA = viral inhibitory activity)

Increased CD160/PD1 co-expression on CD8+ TEMRAs of suppressors at baseline

In order to look for correlates of VSC with so-called 'exhaustion markers' at baseline, we phenotyped non-stimulated, rested CD8+ T cells using the immune check point panel. Interestingly, CD8+ T cells from suppressors only showed a significantly higher CD160/PD1 co-expression in the terminally differentiated effector memory subset (TEMRA), defined as CCR7-CD45RA+, as compared to non-suppressors (Fig. 4A). To identify CD8+ T cell memory subsets

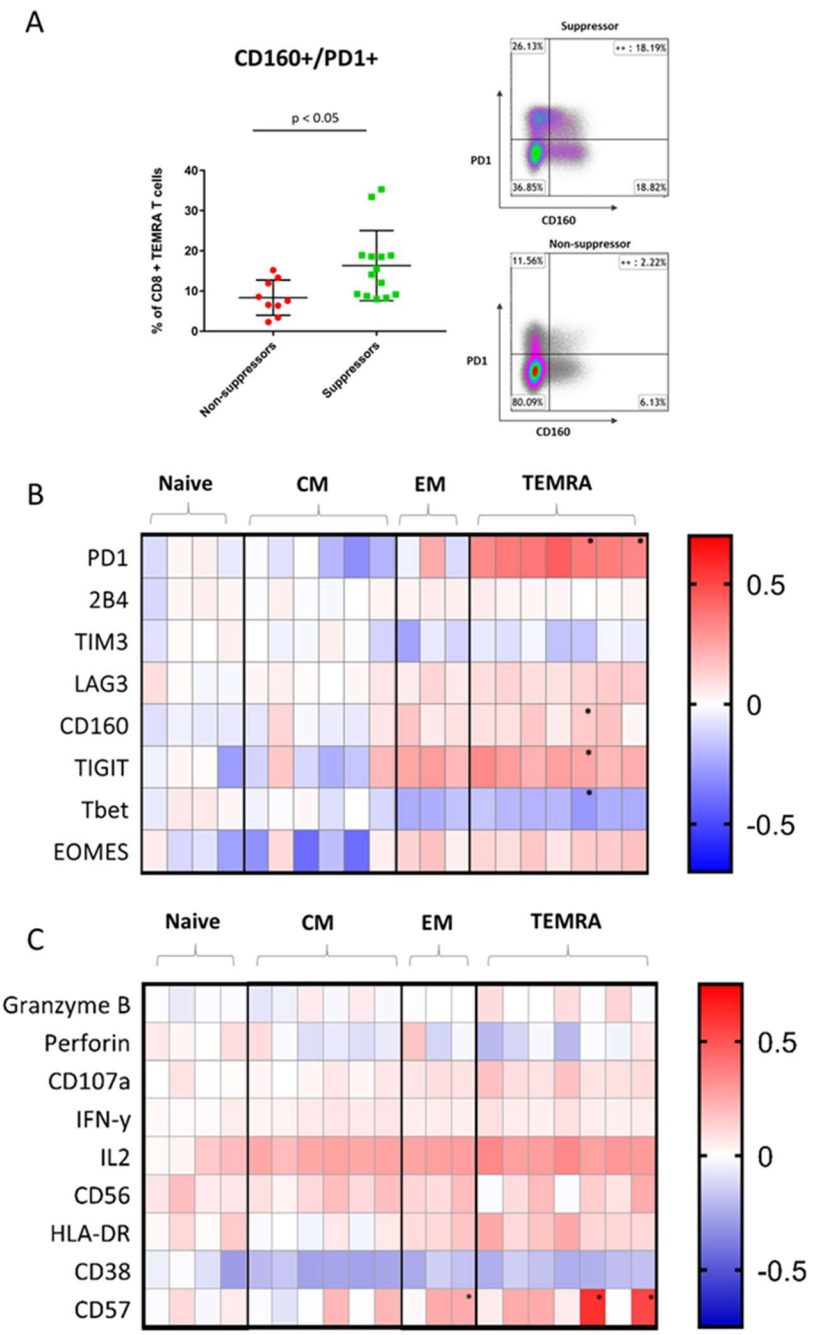


Figure 4. (A) Manual (bivariate gating) analysis showing percentage of PD1/CD160 double positive CCR7-CD45RA+ (TEMRA) CD8+ T cells in suppressors and non-suppressors. Representative flow cytometry plots of suppressors and non-suppressors are given on the right. **(B,C)** SPADE cluster analysis based on CD45RA and CCR7 expression of CD8+ T cells. The heat map depicts for each marker the fold change in MFI of suppressors versus non-suppressors for the 'immune exhaustion' **(B)** panel and the 'cytotoxicity/activation' panel **(C)**. Significant differences ($p < 0.05$) are marked with asterisks (p-values are not adjusted for multiple testing). (CM = central memory; EM = effector memory; TEMRA = terminally differentiated effector memory T cells)

with different expression patterns of exhaustion markers between suppressors and non-suppressors, we ran a SPADE analysis based on CD45RA and CCR7 expression (Fig. 4B). Four, six, three and seven subsets were identified in the naïve, central memory (CM), effector memory (EM) and TEMRA CD8+ T cells respectively (Fig. 4B). The comparison of suppressors versus non-suppressors identified a cluster with significantly higher PD1/CD160/TIGIT co-expression but lower Tbet expression in suppressors. Another cluster showed significantly higher expression of PD1 only. These differences were however only significant without Bonferroni-Holm's corrections of p-values.

Increased CD57 expression on CD8+ T cell memory subsets of suppressors after peptide stimulation

In order to look for correlates of VSC with activation and cytotoxicity markers, we phenotyped CD8+ T cells after 6 hours of stimulation with an HIV-1 consensus B *gag* peptide pool. Using the SPADE cluster analysis described above, we observed significantly higher CD57 expression (without Bonferroni-Holm's correction) in one EM and two TEMRA subpopulations (Fig. 4C) in suppressors. No higher expression was observed for IFN-γ, IL-2, granzyme, perforin or CD107a between suppressors and non-suppressors among the memory subsets after six hours of peptide stimulation.

HLA-DR expression on CD8+ T cells of suppressors is increased early in co-culture

Finally, we phenotyped CD8+ T cells in a small available subset of six patients 18 and 42 hours after the start of co-culture to assess their activation and cytotoxicity phenotype. We found that suppressors had a higher ($p < 0.13$) HLA-DR expression 18 and 42 hours after the start of co-culture as compared to non-suppressors (Fig. 5A). There were no differences in expression of IL-2, granzyme B and perforin between the two groups. Furthermore a slightly higher but

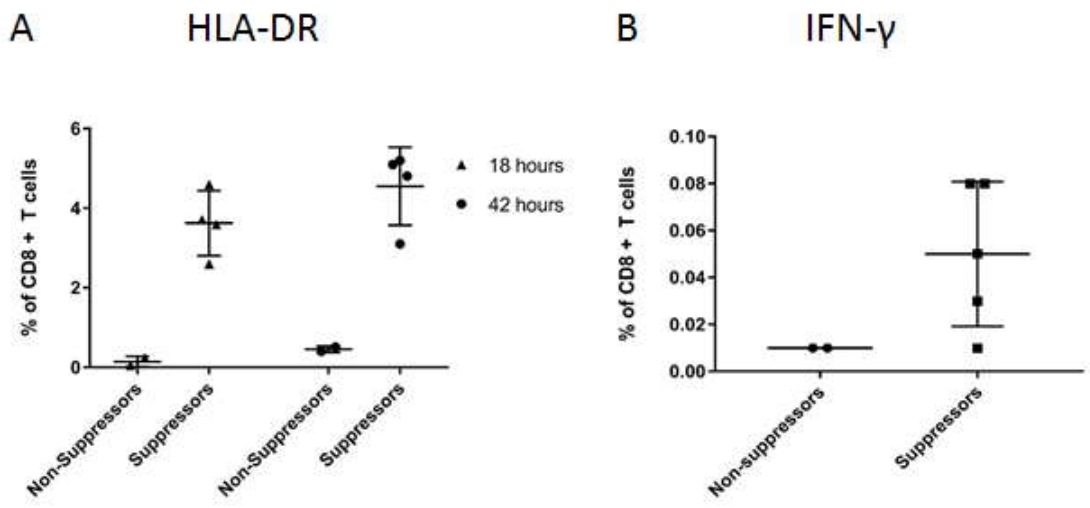


Figure 5. (A) Percentage of HLA-DR positive CD8+ T cells 18 and 42 hours after the start of CD4+ T cell/CD8+ T cell co-culture in suppressors and non-suppressors. **(B)** Percentage of IFN-γ positive CD8+ T cells 42 hours after the start of CD4+ T cell/CD8+ T cell co-culture in suppressors and non-suppressors.

non-significant IFN-γ response in suppressors was observed after 42 hours of co-culture (Fig. 5B).

Discussion

A better understanding of the anti-viral activity of CD8+ T cells is crucial in the search for a functional cure for HIV. In our study we show that in-vitro VSC of CD8+ T cells from a proportion of progressors under cART can be induced with an HIV specific peptide stimulation. While this induced VSC did not correlate with any clinical parameters or viral reservoir measures, we did observe a correlation with IFN-γ production by PBMCs in response to *gag* peptide stimulation. Importantly, we identified specific subsets of TEMRA CD8+ T cells with higher PD1+CD160+ co-expression at baseline, higher CD57 expression after peptide stimulation and higher HLA-DR expression during co-culture with infected CD4+ T cells in patients with higher VSC.

We presume that HIV specific memory CD8+ T cells are expanded during the seven day peptide stimulation and revert from a resting to a more functional effector phenotype, thereby increasing VSC. This idea of specific expansion, rather than the induction of new or non-specific responses is further supported by (a) the strong correlation between VSC of rested versus peptide stimulated CD8+ T cells, (b) the very low background of VSC in healthy controls and (c) the failure to induce VSC in HIV non-subtype B infected patients (Fig. 1).

VSC has been described to correlate with plasma viral load [112, 132], suggesting the importance of antigen exposure to keep up cellular anti-HIV immunity. The five patients with low but detectable VLs did have higher VSC with and without peptide stimulation as compared to the patients with undetectable VLs, although this difference was not significant. Similarly, we hypothesized that reservoir size and on-going viral transcription, presumably resulting in antigen presentation and boosting of the immune system, could correlate with CD8+ T cell VSC. Nevertheless, we did not find any correlations with HIV-1 DNA or RNA. It is possible however that the small range in unspliced cell-associated RNA levels in our cohort [IQR: 0.2 – 10.7 cps per million PBMCs] did not allow to observe significant differences in VSC.

Several groups have shown the importance of polyfunctionality and the lack of correlation between VSC and IFN-γ responses of CD8+ T cells [114, 122, 128]. Nevertheless, we observed that the amount of IFN-γ produced in response to an HIV peptide stimulation accumulated over a period of seven days in the supernatant significantly correlated with subsequent VSC (Fig. 3A, $p < 0.01$). Suppressors also had a significantly higher ratio of TNF-α/IL-10 production (Fig. 3D, $p < 0.05$).

Phenotypic data demonstrated higher numbers of PD1+CD160+ double positive CD8+ TEMRA T cells in suppressors. Expression of such immune checkpoint (IC) markers has been linked to

T cell exhaustion in the context of chronic disease with sustained antigen exposure, resulting in a gradual loss of effector functions [232-237]. TEMRA cells on the other hand are also linked to chronic antigen exposure and make up an aged subset of EM cells, characterized by a decreased proliferation potential but strong effector capacities [238]. While the observed positive correlation with VSC therefore seems paradoxical, the following points need to be taken into consideration.

Earlier studies have shown that CD8+ T cells remain responsive despite chronic infection [97, 239]. In lymphocytic choriomeningitis virus infection phenotypically exhausted memory CD8+ T cells control viral infection despite continuous expression of PD1 [240]. During untreated HIV-1 infection increasing viral loads correlate with declining numbers of polyfunctional CD8+ T cells and increased expression of IC markers [136, 241, 242]. During cART the decay of PD1 on T cells coincides with the decline of immune activation and viral load but are never restored to levels of HIV negative individuals [241, 243]. Expression of IC markers has been reported on CD8+ T cells of ECs, suggesting residual viral replication rather than dysfunctionality of the immune response [244]. In addition, ECs harbor highly effective cytolytic subsets in CD8+ T cells expressing CD160 and 2B4 [245]. Taken together, this data supports the idea that a range of exhausted states may exist within functional effector subsets. Accordingly, suppressors were shown to have a larger subset of PD1+CD160+ CD8+ T cells of the TEMRA compartment as compared to non-suppressors, possibly representing more functional and cytolytic cells able to suppress viral replication.

Furthermore, a higher CD57 expression was observed in EM and TEMRA subsets of suppressors as compared to non-suppressors. These respective subsets have typically two profiles: a highly cytotoxic and a poorly replicative one [246, 247]. In addition, CD57 was

previously suggested as a marker of proliferative history, and more recently as a maturation marker for cytotoxic HIV specific CD8+ T cells with a more terminally differentiated phenotype in ECs [150, 247, 248]. Together, these findings are suggestive of terminal differentiation as a key factor for a potent anti-viral response [249]. Finally, a higher HLA-DR expression in CD8+ T cells was observed in suppressors during co-culture with autologous CD4+ T cells, suggesting that a distinct activation state of fully differentiated memory subsets in suppressors might explain VSC.

While we have identified a phenotypical difference between CD8+ T cells from patients with and without in-vitro viral suppression, further research is needed to determine whether these distinct subsets are in fact responsible for suppressing viral replication.

Supplementary methods

Inclusion criteria for HIV-1 infected subjects:

- Treated with conventional cART.
- Present a routinely "undetectable" plasma VL (< 20 copies/ml) for >6 months.
- They should be in good general condition.
- They should present with actual CD4 T cell count > 300/µL.
- Adult men and women are eligible, but women should not be pregnant.
- Informed consent must be given.

Details of the human species reactive antibodies included in the "cytotoxicity/activation" and "immune checkpoint" panels:

Cytotoxicity/activation panel: BD Biosciences (San Jose, CA): CD3 (BUV496, clone UCTH1); CD4 (BUV395, clone SK3); CD8 (BUV805, clone SK1); CD107a (BV421, clone H4A3); IL2 (BV605; clone 5344.111); CD56 (BV786; clone NCAM16.2); IFN-γ (FITC, clone 4S.B3); Granzyme B (PE, clone GB11). Biolegend (San Diego, CA): CD45RA (Pacific Blue, clone HI100); HLA-DR (BV510, clone L243); CD38 (PerCp/Cy5.5, clone HIT2); CCR7 (PECF594; clone 6043H7); Perforin (PE-Cy7, clone BD48); CD57 (APC, clone HCD57); Zombie NIR fixable viability kit (APC-Cy7).

Immune checkpoint panel: BD Biosciences (San Jose, CA): CD3 (BUV496, clone UCTH1); CD4 (BUV395, clone SK3); CD8 (BUV805, clone SK1); CD28 (BV605, clone 28.2); CD27 (Bv650, clone MT271); TIM-3 (BV711, clone 7D3); Invitrogen (Waltham, MA): EOMES (PE-Cy7, clone WD1928). eBioscience (Waltham, MA, USA): CD160 (FITC, BY55); LAG-3 (efluor 710, clone 3DS223). Biolegend (San Diego, CA): PD1 (BV421, clone EH12.1); 2B4 (Pacific Blue, clone C1.7); CD45RA (Bv786, clone HI100); Tbet (PE, clone 4B10), CCR7 (PECF594; clone 6043H7); TIGIT (APC, clone A15153G); Zombie NIR fixable viability kit (APC-Cy7).

Acknowledgments

We would like to thank Maartje Van Frankenhuijsen, Odin Goovaerts and Meryam Krit from the ITM in Antwerp as well as Karen Vervisch, Maja Kiselinova and Eva Malatinkova from Ghent University for their help in the clinic and laboratory. We would like to thank the Blood Transfusion Center of The Red Cross in Mechelen for providing the buffy coats of healthy donors and the NIH AIDS Reagent Program for providing the HIV-1 Consensus B Gag peptide pool and anti-human CD3/8 bi-specific monoclonal antibodies.

Chapter 6 - iHIVARNA – therapeutic vaccination with TriMix / mRNA to achieve delayed viral rebound

Author's contributions: The author of this thesis contributed to the work presented underneath by setting up of, testing patient samples with, and analyzing results from the ultra-sensitive plasma viral load assay (phase 1 and 2) and the viral inhibition assay (phase 2). The author also contributed to the writing of the lab analytical plan of the phase 2 study, participated in the general assembly meetings and co-authored both phase 1 and 2 manuscripts.

Co-author's contributions: The responsibilities of the different aspects of the iHIVARNA trial are listed underneath:

- IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona): patient recruitment; sponsor of phase 1 of the study; first-author of the phase 1 paper; IFN-γ ELISpot analyses
- IRSICAIXA (Institut de Recerca de la Sida, Badalona): patient recruitment; droplet digital PCR measurements of HIV-1 DNA and cell-associated RNA
- VUB (Vrije Universiteit Brussel, Brussels): patient recruitment
- EMC (Erasmus Medical Center, Rotterdam): patient recruitment; sponsor of phase 2 of the study; first-author of the phase 2 manuscript; transcriptome analysis (not presented in this thesis)
- eTheRNA: production of the mRNA vaccine

Abstract

While combination antiretroviral therapy is not curative, a small proportion of HIV-1 infected classic progressor patients has been observed to achieve functional cure, i.e. a durable state of viral remission after treatment interruption. This 'post-treatment control' (PTC) has been associated to small reservoir size, low proviral transcriptional activity and to HIV-suppressive CD8+ T cell responses. One of the current strategies to achieve PTC is to induce such CD8+ T cell responses through therapeutic vaccination.

Within the iHIVARNA consortium, we aimed to test a novel mRNA vaccine (coding for the vaccine itself and the adjuvant) combining three innovative strategies: (1) direct intranodal injection of naked mRNA, which has been observed to induce better immune responses, (2) a rational selection of immune subdominant HIV peptides which are associated to protective responses, and (3) a potent adjuvant composed of three dendritic cell activation stimuli, named TriMix. To this end, we performed a phase 1 dose escalation clinical trial to determine the safety, and a phase 2a trial to determine the immunogenicity of the vaccine.

After the end of the second trial however, a serious error was discovered in the mRNA construct. Namely, a second start codon was found upstream of the original start codon of the vaccine open reading frame. The presence of this upstream open reading frame likely interfered with the efficient translation of the vaccine mRNA. As the degree of this interference is unknown, it remains unclear to what extent the vaccine mRNA has been expressed.

As a result, we were not able to determine the safety or immunogenicity of our vaccination strategy. Further studies using the same concepts remain indicated.

Introduction

The advent of combination anti-retroviral therapy (cART) has strongly reduced the number of deaths and AIDS related illnesses. However, cART is not able to completely eliminate the virus since pro-viruses which are stably integrated in latently infected CD4+ T cells remain untouched by therapy [39, 176]. In addition, cART does not restore HIV-suppressive CD8+ T cell responses [250] and consequently has to be taken life-long, with long term adverse effects, risk of drug resistance development and important costs as a result.

A small proportion of patients, however, is able to control viral replication after a period of therapy, a phenomenon termed 'post treatment control' (PTC). This state of 'functional cure' has best been correlated with small reservoir size (low HIV-1 DNA), low levels of pro-viral transcriptional activity (low spliced and unspliced cell-associated RNA) and potent HIV-suppressive CD8+ T cell responses [143, 251, 252]. As a result, one of the current strategies to achieve functional cure consists of inducing strong and protective HIV-suppressive CD8+ T cell responses by therapeutic vaccination.

In this regard, it was previously shown that HIV-suppressive CD8+ T cell responses can be induced *in vivo* by vaccination, using dendritic cells (DC) loaded with HIV antigens. Indeed, increases in magnitude and breadth of HIV-specific IFN-γ responses as well viral inhibitory activity (VIA) were observed post-vaccination as compared to pre-vaccination [253]. Another clinical trial showed strong reductions in peak viral load in DC-vaccinated patients after treatment interruption. Nevertheless, none of the patients were able to suppress viral replication completely, indicating that the induced immune responses were insufficiently strong [254]. In the iHIVARNA consortium, a number of novel vaccination design strategies have been combined in order to induce stronger immune responses.

a. Partner institution 'Vrije Universiteit Brussel' (VUB, Brussels, Belgium) obtained data from mouse tumor models which suggests a better induction of tumor-specific immune responses by **direct intranodal injection** of a tumor antigen mRNA based therapeutic vaccine as compared to intradermal administration of mRNA electroporated DCs [255].

b. Partner institution 'IRSICAIXA' (Badalona, Spain) performed a large-scale screening of 950 untreated HIV-1 positive patients for IFN-γ responses to sets of 410 overlapping HIV-1 peptides and correlated these responses to the patients' viral load. Responses against 26 CTL epitopes covered by these overlapping peptides (in *gag, pol, vif, nef*) were identified as significantly **correlated to lower viral loads** in untreated patients and are therefore considered to be protective responses [83]. Based on these data, IRSICAIXA has designed an immunogen sequence, named HIVACAT T cell immunogen (HTI), which has been proven to be broadly immunogenic in preclinical animal studies [256].

c. Since naked antigenic mRNA alone might not be able to fully activate and mature DCs, partner institution VUB has developed a **mix of mRNA encoding adjuvant activation stimuli**, including CD40 ligand (CD40L), a constitutively active variant of Toll-like receptor (caTLR) 4 and CD70, named TriMix. DCs electroporated with TriMix-mRNA have been shown to enhance T-cell stimulatory capacity [257, 258].

A preclinical evaluation of this strategy in both human and mouse models, confirmed that the mRNA vaccine is indeed able to activate DCs and induce HIV-specific T cell responses [256]. Consequently, the final iHIVARNA vaccination strategy to be used in clinical trials consisted of three direct intranodal injections of mRNA coding for TriMix and HTI to ultimately try and achieve functional cure.

A phase 1 dose escalation study was conducted with the primary objectives to assess feasibility and safety of the iHIVARNA vaccination strategy and secondary objectives to have a preliminary indication of the immunogenicity of the vaccine and its effect on the viral reservoir. Based on the proven, albeit moderate, immunogenicity of the highest dose of the vaccine from phase 1, a phase 2a proof-of-concept clinical trial including a treatment interruption was conducted. The primary objectives of this second phase were to evaluate the immunogenicity and safety of the vaccine while the secondary objectives included the effect of vaccination on time to viral rebound, CD8+ T cell mediated HIV suppressive capacity (VIA) and the size and transcriptional activity of the viral reservoir.

Disclaimer: After completion of the phase 2a trial, a serious **problem** in the mRNA construct was discovered. Namely, the construct was found to contain an **extra start codon** upstream of the HTI open reading frame. The presence of this extra start codon is likely to significantly **interfere** with translational initiation from the original start codon. As a result, it is unclear to what extent the mRNA vaccine has been **expressed**, if at all, in both phase 1 and phase 2a trials.

The reader of this work should keep the above in mind when interpreting the immunogenicity observed in these trials. This major limitation will be further elaborated on in the discussion section of this chapter.

Subjects, materials and methods

Phase 1 patients and samples

An open-label, dose escalating phase 1 clinical trial was performed in a single center (IDIBAPS, Barcelona, Spain). Twenty-one chronically HIV-1 infected patients on cART, with suppressed viremia (<50 cps/mL), current CD4+ T cell count >450/µL and nadir CD4+ T cell count >350/µL were included. Exclusion criteria included active hepatitis B or C infection and pregnancy. Patients were screened for participation at week -4 after which three inguinal intranodal doses of mRNA were administered at weeks 0, 2 and 4. Each patient was followed-up for 24 weeks (Fig. 1).

The 21 patients were divided into five groups according to the dose escalation scheme detailed in Table 1. Enrollment of the next dose level group was only allowed if less than half of the patients of the previous group developed a dose limiting toxicity (DLT). If more patients developed a DLT, the study could either be terminated or patients could be recruited in a lower dose level group. In addition, each patient was monitored for at least one week before the next patient was enrolled. Similarly, each group was monitored for at least two weeks after the last vaccination before the next dose level group was enrolled.

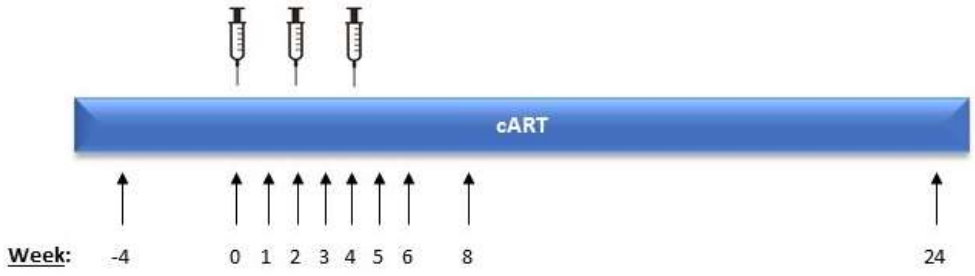


Figure 1. Overview of phase 1 of the study. At week -4, patients were screened for enrollment. Three intranodal injections were administered at respectively week 0, 2 and 4. Patient follow-up visits are indicated with arrows, injections are indicated with syringes. (cART = combination antiretroviral therapy)

Table 1. Phase 1 patient groups

Group	Patients	TriMix mRNA (µg)	HTI mRNA (µg)
1	3	100	/
2	3	300	/
3	3	300	300
4	6	300	600
5	6	300	900

Dose escalation scheme for five patient groups included in the phase 1 iHIVARNA clinical trial. TriMix mRNA codes for CD40L, a caTLR4 and CD70. HTI mRNA codes for the HIVACAT immunogen sequence.

Phase 2 patients and samples

After completion of the first phase, a double-blinded, multi-centric phase 2a clinical trial was performed in five centers (Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona; Institut de Recerca de la Sida, Badalona; Institute of Tropical Medicine, Antwerp; Vrije Universiteit Brussel, Brussels; Erasmus Medical Center, Rotterdam). For this randomized placebo controlled double blind study, thirty-eight chronically HIV-1 infected patients on cART were screened. Candidates with suppressed viremia (<50 cps/mL), current CD4+ T cell count >450/µL and nadir CD4+ T cell count >350/µL were enrolled after screening on week -4. Again, exclusion criteria included active hepatitis B or C infection and pregnancy. Three inguinal intranodal doses of placebo or mRNA were administered at weeks 0, 2 and 4 (Fig. 2). Randomization of the patients resulted in 15 patients receiving Water for injection (WFI, a physiological salt solution) (Placebo), 15 patients receiving 300µg of TriMix mRNA only (TriMix) and 40 patients receiving 300µg of TriMix mRNA and 900µg of HTI mRNA (HTI + TriMix). This schedule is summarized in Table 2.

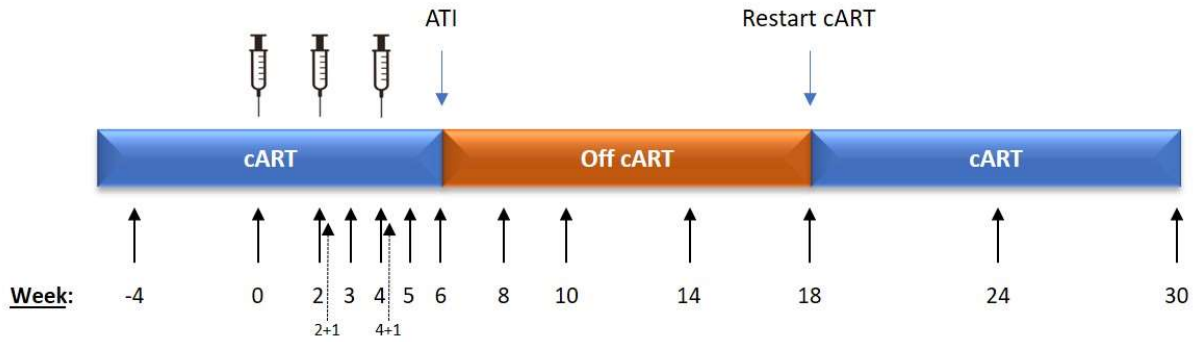


Figure 2. Overview of phase 2a of the study. Time points at which blood was drawn for plasma and PBMC separation are indicated with arrows. Three intranodal injections were administered at respectively week 0, 2 and 4. cART was interrupted at week 6 and restarted when treatment resumption criteria were reached or 12 weeks after ATI (week 18).

Table 2. Randomization of 33 patients included in the phase 2a iHIVARNA clinical trial.

Group	Patients	TriMix mRNA (µg)	HTI + TriMix mRNA (µg)
Placebo	15	/	/
TriMix	15	300	/
HTI + TriMix	40	300	900

Two weeks after the third (= last) vaccination (i.e. week 6), patients with undetectable viral load and CD4+ T cell count > 450/µL underwent an analytical treatment interruption. Patients were monitored at the time points indicated on fig. 2 for 30 weeks or 12 weeks after treatment resumption. Treatment resumption criteria included a decline of CD4+ T cell count <400/µL, a loss of CD4+ T cells >30% from baseline, appearance of any clinical symptoms related to cART discontinuation, a detectable viral load at two consecutive measurements two weeks apart or at the decision of the treating physician.

Vaccine

The vaccine consisted of mRNA coding for the HIVACAT T-cell Immunogen (HTI). This sequence comprised 16 joined fragments of 10-70 amino acids each, coding for conserved and immune subdominant epitopes in Gag, Pol, Vif and Nef. The adjuvant (TriMix) consisted of mRNA

coding for CD40L and a constitutively active variant of TLR-4 to induce dendritic cell maturation, and CD70 to support activated T-cell survival and proliferation.

Immunogenicity assessments

To assess immunogenicity of the vaccine, IFN-γ ELISpot responses as a primary endpoint and viral inhibitory activity as a secondary endpoint were measured on cryo-preserved peripheral blood mononuclear cells (PBMC).

IFN-γ ELISpot

Quantification of HIV-specific CD8+ T-cell responses by IFN-γ ELISpot was performed at IDIBAPS (Barcelona) on the following time points:

- phase 1: weeks 0 (i.e. day of first immunization), 4, 6, 8 and 24.
- phase 2: weeks -4 (i.e. 4 weeks before first immunization), 0, 4, 6, 10, 18 and 30.

Briefly, cryo-preserved PBMCs were thawed and rested overnight at 37°C in complete medium. Next, 100,000 PBMCs were stimulated with peptide pools (1µg of each single peptide) in 100µL of complete media (RPMI plus 10% FCS) in duplicate conditions. To evaluate HIV-specific CD8+ T-cell responses against the whole HIV proteome, sets of overlapping HIV peptide pools were used (10 different pools containing from 5 to 22 15-mer peptides, overlapping by 11 peptides, which matched the HTI immunogen ("IN") and 8 pools ranging from 62 to 105 15-mer peptides covering NIH consensus subtype B HIV sequences not located within HTI ("OUT")). Medium alone in triplicate was used as negative control. Stimulation with phytohemagglutinin PHA-P (1µg/ml) and CEF (CMV, EBV and Flu) pools were used as positive controls. Results are expressed as the mean number of spot forming cells (SFC)/10^6 cells from duplicate wells. The following criteria were used to define the technical validity and positive responses: PBMC viability had to be >80%; the assay background (PBMCs + medium) had to

be <50 SFC/10^6 PBMC; positive responses against PHA-P had to be above 500 SFC/10^6 PBMC; and ELISpot responses were considered positive in case of >50 SFC/10^6 PBMCs and number of SFC/10^6 PBMCs at least ≥2-fold over the medium control.

Viral inhibitory activity

Viral inhibitory activity (VIA) was measured with a viral inhibition assay at the ITM (Antwerp). VIA was only measured in phase 2a patients at weeks -4, 0 and 4.

For the preparation of CD4+ T target cells, autologous PBMCs were stimulated during seven days at 37°C 7% CO_2 in RPMI 2.5% human serum (HS, A&E Scientific, Belgium), IL-2 (500IU/mL, Gentaur, Kampenhout, Belgium) and anti-human CD3/8 bi-specific monoclonal antibody (1µg/mL, NIH AIDS Reagent Program). For the preparation of stimulated CD8+ T effector cells, autologous PBMCs were incubated during 7 days at 37°C 7% CO_2 in RPMI 2.5% HS and 28.6µg/mL (200ng/mL per peptide) of the HTI "IN" peptide pool. For the preparation of non-stimulated CD8+ T effector cells, an extra aliquot of autologous PBMCs was thawed one day before the start of CD4+/CD8+ T cell co-culture and rested overnight at 37°C 7% CO2 in RPMI 2.5% HS.

On day 0 of the VIA assay, CD4+ T target cells were enriched by negative selection magnetic beads (Miltenyi Biotech, San Diego, CA, USA) from the anti-CD3/8 mAb stimulated PBMCs. Stimulated as well as non-stimulated overnight rested CD8+ T effector cells were enriched from the HIVACAT peptide pool stimulated PBMCs and overnight rested PBMCs respectively, by negative selection. Cell purities of enriched CD4+ and CD8+ T cells >90% were confirmed by staining for CD3 (FITC, Clone OKT3), CD4 (PE, clone SK3), CD8a (APC-eFluor, clone SK1) and reading on a BD FACSVerse (Beckton Dickinson, New Jersey, USA).

Enriched CD4+ T cells were incubated with HIV III_B (NIH AIDS Reagent Program) at a multiplicity of infection of 0.001 for 3 hours at 37°C 7% CO_2. Infected target cells were washed three times and resuspended at 10^6 cells/mL in RPMI 2.5% HS 500IU/mL IL-2 and cultured in triplicate in flat bottom 96-well plates (VWR, Leuven, Belgium) at 10^5 cells/well, alone (positive control) or in co-culture with stimulated or rested enriched CD8+ effector T cells at an effector-to-target ratio of 0.1:1 and 2:1. Culture medium was refreshed at days 2, 6 and 9. The levels of HIV-1 p24 antigen in the supernatant were determined at day 13 by in-house p24 ELISA [226]. Log inhibition values were calculated on day 13 as $\log_{10}$(p24 without CD8) – $\log_{10}$(p24 with CD8).

Reservoir assessments

To assess the effect of the vaccine on the size and transcriptional activity of the viral reservoir as well as on the level of residual viremia, HIV-1 DNA, cell-associated unspliced RNA (US-RNA) and ultra-sensitive plasma viral load (usVL) were determined.

HIV-1 DNA and US-RNA

HIV-1 DNA and US-RNA levels were quantified in peripheral CD4+ T cells on the following time points:

- phase 1:
    - all groups: weeks 0, 4, 6, 8 and 24
    - groups 4 & 5 only: week 2 +1 day, week 3, week 4 +1 day and week 5
- phase 2:
    - DNA and US-RNA: week 0, week 2 +1 day, week 4, week 6, week 18, week 30
    - US-RNA only: week 2 +1 day, week 3, week 4 +1 day, week 5

Both DNA and US-RNA were measured using droplet digital PCR (ddPCR) with two different primer sets (5'LTR and *Gag*) to avoid mismatching, as described previously [259]. HIV-1 DNA was normalized relative to the housekeeping gene Ribonuclease P protein subunit p30 (RPP30) and expressed as copies per million CD4+ T cells. HIV-1 US-RNA was normalized relative to the housekeeping gene TATA-binding protein (TBP) and expressed as copies.

Ultra-sensitive plasma viral load (usVL)

The ultra-sensitive plasma viral load (usVL) assay was performed in groups 4 and 5 of phase 1 and all patients of phase 2 on the following time points:

- phase 1: weeks 0, 2+1day, 3, 4, 4+1day, 5, 6, 8 and 24
- phase 2: weeks -4, 0 and 6

Five mL (phase 1) or 10mL (phase 2) of EDTA plasma was centrifuged at 170,000 x g for 1h at 4°C in phase 1 and 2 respectively. Tubes were equilibrated with Tris buffered saline (50 mM Tris-Cl, pH 7.6; 150 mM NaCl) to a final volume of 12mL. After centrifugation, 11.20mL of supernatant was carefully aspirated and discarded. The pellet was thoroughly resuspended in the remaining 800µL and tested for viral load with the Cobas® HIV-1 test on the Roche Cobas® 4800 system. To account for the concentration of the virus, the obtained result was multiplied by a factor 0.16 (0.8/5) and 0.08 (0.8/10) for phase 1 and 2 respectively.

Interim analysis

After recruitment of half of the participants, an interim analysis was performed for futility on the primary endpoints of the study, i.e. immunogenicity and safety. Immunogenicity results at week 6 of the study (i.e. 2 weeks after the last vaccination) were probed to show a 0.7log increase in the HTI-TriMix arm, compared to the Placebo-arm (primary end point in study

protocol). In case standard test statistics for comparing two means was smaller than 2.23, the trial was stopped for futility.

Statistical analyses

The sample size for the phase I study was the minimum required to achieve study objectives as stated in 'Guideline on Requirements for First-in-man clinical trials for potential high-risk medicinal products' (EMEA/CHMP/SWP/28367/2007). The sample size for the phase 2 study was originally calculated at 70 (15 placebo, 15 TriMix, 40 HIVARNA) but based on the poor immunogenicity results an interim analyses was decided for, after vaccination of 33 participants. The futility analysis dictated the stop of further inclusion of volunteers in the phase II clinical trial.

Safety analyses were descriptive and safety endpoints were described and summarized by number and percentage of adverse events (AEs) and grading. AEs were stratified into unrelated/unlikely/possibly/probably/definitely related to the vaccine. Differences in HIV-1 specific CD8+ T cell responses, HIV-1 DNA, HIV-1 US-RNA, usVL and VIA between two longitudinal determinations in the same individuals were assessed by Wilcoxon Signed-Rank tests.

Results

Phase I - Clinical Trial of an Intranodally Administered mRNA Based Therapeutic Vaccine Against HIV-1 Infection

Vaccination with TriMix and HTI mRNA in HIV-1 infected patients on cART is safe

All 21 participants received 3 doses of mRNA (TriMix and/or HTI) by inguinal intranodal injection according to the planned dose escalating scheme (Methods, table 1) since none of the participants experienced dose limiting toxicities. All participants completed the entire

study period of 24 weeks of follow up after the first vaccination. Table 3 summarizes the clinical characteristics of all participants.

Administration of Trimix and/or HTI mRNA was generally safe. No serious adverse events (AE) were recorded. Over the entire follow-up period, a total of 32 AEs were recorded, of which only one (3%) grade 3 event which was not related to the vaccination. Two (6%) AEs were probably or definitely related, 14 (44%) AEs were possibly related to the vaccination (Table 4).

Table 3. Clinical characteristics of phase I participants (adapted from Leal. et al 2018)

N = 21	Group 1 (n=3)	Group 2 (n=3)	Group 3 (n=3)	Group 4 (n=6)	Group 5 (n=6)
Median age (IQR)	48 (48-51)	51 (48-51)	45 (37-46)	55 (53-57)	47 (43-55)
Male	2	2	2	4	6
MSM	2	2	2	4	6
Heterosexual	1	1	1	0	0
IVD user	0	0	0	2¥	0
HepC infection	0	0	0	2*	0
Median baseline CD4 count (IQR)	762 (686-770)	726 (716-824)	821 (741-957)	829 (680-1124)	904 (868-1071)
Median CD4 count at week 6 (IQR)	672 (663-683)	938 (784-993)	643 (589-1040)	819 (640-928)	956 (805-1053)

¥ former intravenous drug user *both HCV infections cured: 1 spontaneously (2009), 1 treated with sofobusvir/daclatasvir (2015) MSM: Men who have sex with men. IQR: Interquartile range.

Vaccination with TriMix and HTI induces modest increases in vaccine-specific T cell responses

In patients receiving TriMix only (groups 1 and 2) and patients receiving Trimix in combination with the lowest doses of HTI (group 3 and 4), no increases in T-cell responses were observed against HTI-spanning peptides ('IN'), nor against HIV peptides not included in the HTI sequence ('OUT'). In contrast, patients receiving TriMix in combination with the highest dose of HTI (group 5) had slightly increased levels of T-cell responses against 'IN' peptides two weeks after the last vaccination as compared to baseline. No such increase in response was observed against 'OUT' peptides (Fig. 3).

Table 4. Total adverse events in phase I participants classified by severity and relationship with the vaccination (adapted from Leal. et al 2018)

Variable	Value	Val	I	II	III	IV	V	All
Severity	Grade 1	N	3	4	1	9	2	19
		%	75	57	20	82	40	59
	Grade 2	N	1	3	3	2	3	12
		%	25	43	60	18	60	38
	Grade 3	N	0	0	1	0	0	1
		%	0	0	20	0	0	3
	All	**N**	**4**	**7**	**5**	**11**	**5**	**32**
Causal relationship	Definite	N	2	0	0	0	0	2
		%	50	0	0	0	0	6
	Probable	N	0	0	0	0	0	0
		%	0	0	0	0	0	0
	Possible	N	1	4	3	6	0	14
		%	75	57	60	55	0	44
	Not related	N	1	3	2	5	5	16
		%	75	43	40	45	100	50
	Unknown	N	0	0	0	0	0	0
		%	0	0	0	0	0	0
	All	**N**	**4**	**7**	**5**	**11**	**5**	**32**

In addition, the proportion of participants receiving any dose of HTI (groups 3, 4 and 5) responding to the vaccine significantly increased from 31% at baseline to 80% after vaccination. The increase in responders observed in groups 1 and 2 (50% to 67%) was not significant. The most frequent IN-responses were directed against pools containing Gag p17, Gag p24, Gag p15, RT and INT peptides.

Vaccination transiently increases levels of viral transcription and residual viremia, not HIV-1 DNA

As the vaccination might have caused activation of latently infected cells, thereby possibly spurring new infections and increasing the number of infected cells, we determined the effect of the vaccine on the size of the reservoir and its transcriptional activity as measured by total

HIV-1 (pro-viral) DNA and cell-associated US-RNA respectively. No increase in pro-viral DNA was observed in any of the treated groups (Fig. 4A, only arm IV and V are shown). I.e., the vaccinations under the protection of cART did not cause an increase in the size of the viral reservoir.

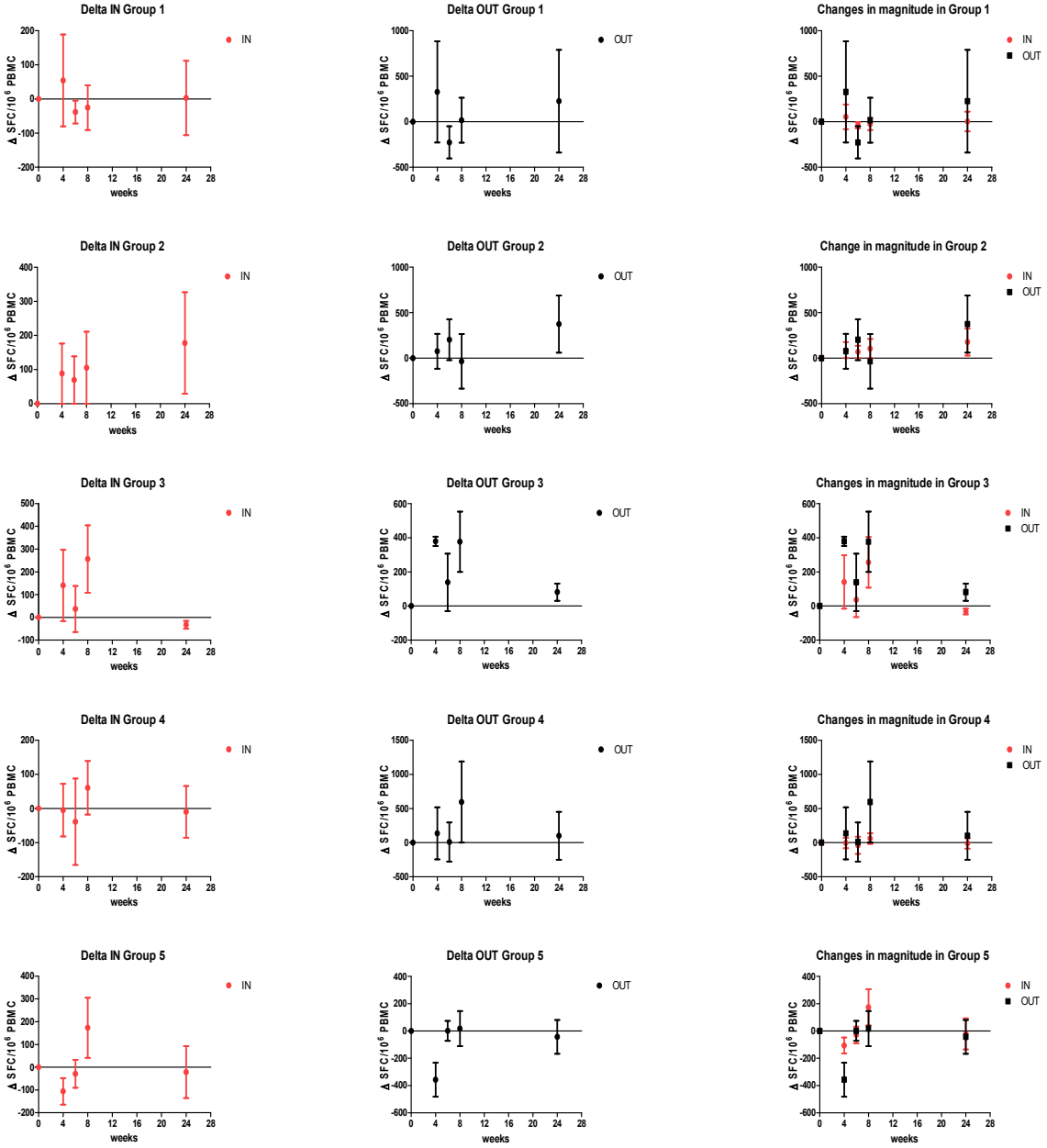


Figure 3. Changes in the magnitude of HIV-1 specific immune responses against IN and OUT pools as measured by ELISpot (adapted from Leal et al. 2018) (SFC = spot forming cells).

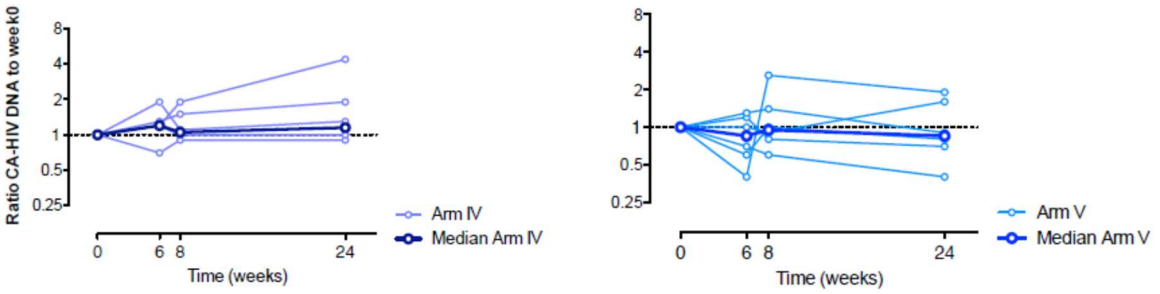


Figure 4A. Impact of the highest doses of iHIVARNA (Groups 4 and 5) on HIV-1 cell-associated (CA) total DNA (adapted from Leal et al. 2018).

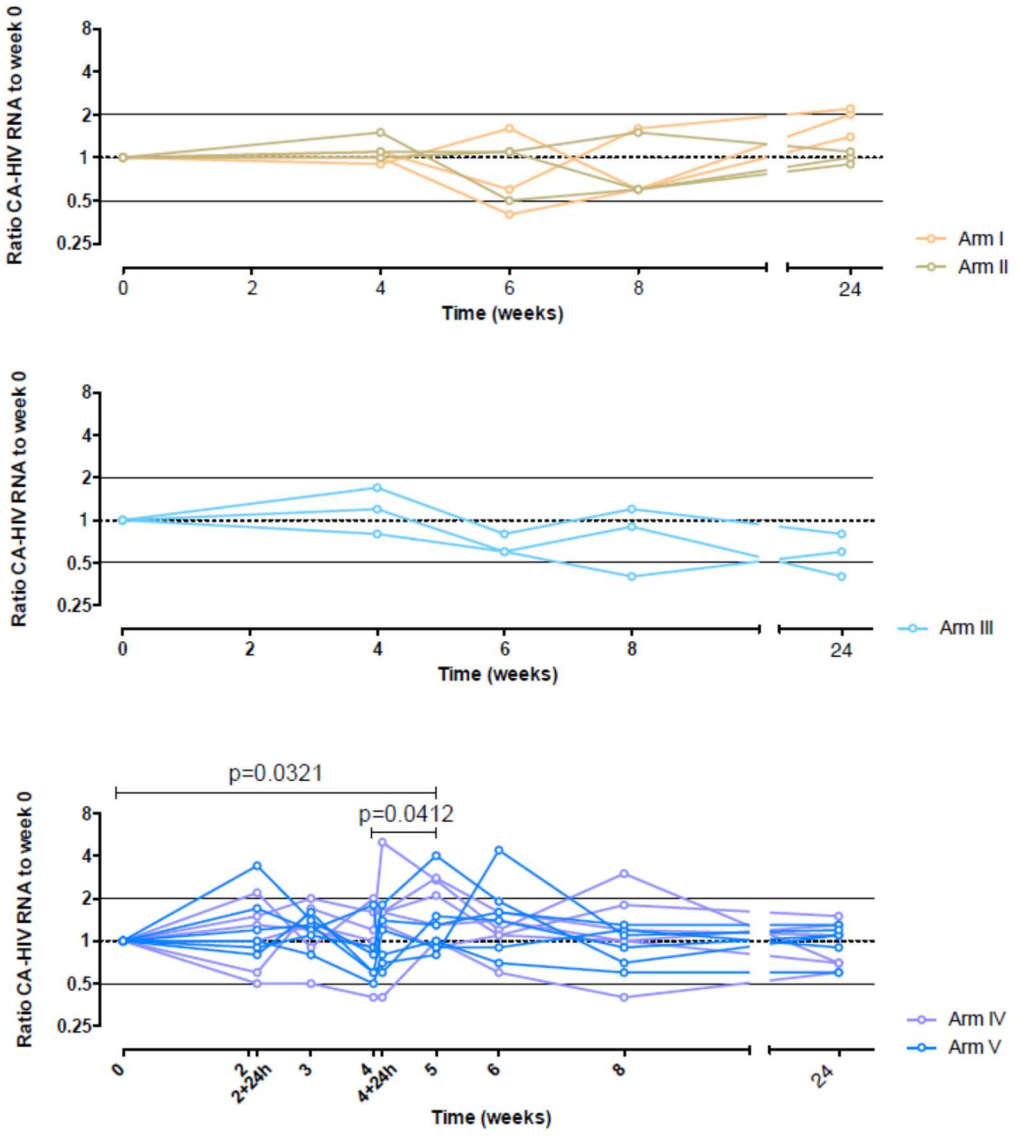


Figure 4B. Impact of TriMix (Groups 1/2) and different doses of iHIVARNA (Groups 3/4/5) on HIV-1 unspliced cell-associated (CA) RNA (adapted from Leal et al. 2018).

However, at the higher doses of TriMix and HTI (groups 4 and 5), transient increases in HIV-1 US-RNA were observed at weeks 4 and 6 as compared to baseline. No such increases were observed in groups receiving TriMix alone (groups 1 and 2) or receiving the lowest dose of HTI (group 3). Nevertheless, levels of US-RNA normalized by week 8 (Fig. 4B). In addition, when comparing week 6 with week 4, patients receiving any dose of HTI (groups 3 ,4 and 5 combined) had a significantly higher ratio of US-RNA than patients receiving TriMix only (groups 1 and 2 combined) ($p < 0.05$).

Finally, we assessed the effect of the vaccine on plasma viral load in participants receiving the highest doses of TriMix and HTI (group 4 and 5). The use of an ultra-sensitive assay with a limit of detection (LOD) of 4 cps/mL allowed us to measure residual viremia (or ultra-sensitive viral load, usVL) below the LOD of commercial assays (20 cps/mL). Two and four weeks after the third and last vaccination (week 6 and 8 respectively), residual viremia was slightly but significantly increased from a mean of 5.6 cps/mL at baseline to 12.1 cps/mL at week 6 ($p < 0.01$) and 9.2 cps/mL at week 8 ($p < 0.05$). By week 24, usVL had normalized to 5.5 cps/mL (Fig. 5).

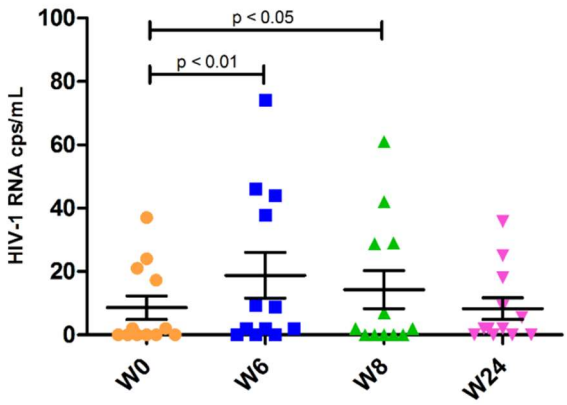


Figure 5. Impact on plasma viral load of the highest doses of iHIVARNA (Groups 4 and 5) as measured by the ultra-sensitive viral load assay at baseline (W0), W6, W8 and W24 (adapted from Leal et al. 2018)

Thus, similar to the US-RNA levels described above, we observed a modest and transient increase in usVL after vaccination. Interestingly, usVL at week 6 was positively and significantly correlated to the increase of elicited T-cell immune responses against HIVACAT peptides (IN) ($p < 0.05$). No such correlation was observed between usVL and responses directed at the rest of the HIV-1 proteome (OUT) (Fig. 6).

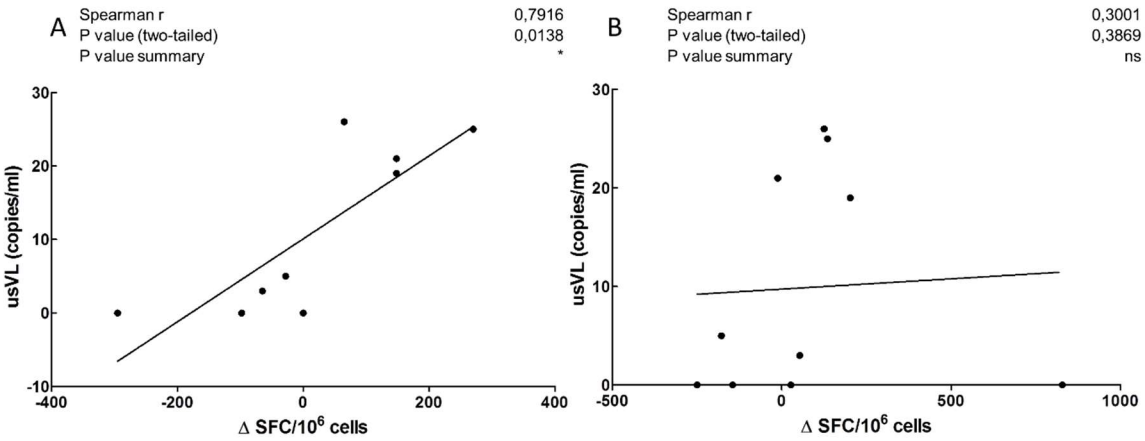


Figure 6. Plots from groups 4 and 5 depicting correlations between the increase of spot-forming cells (SFC) per 10^6 PBMCs at week 6 (compared to baseline) and the ultra-sensitive VL (usVL, copies/ml) at week 6 are shown. The relationship between both parameters showed a significant and positive correlation against peptides included in the HTI immunogen (A) whereas no correlation is observed against peptides outside the insert (B) (adapted from Leal et al. 2018).

Phase 2 - Clinical Trial of an Intranodally Administered mRNA Based Therapeutic Vaccine Against HIV-1 Infection

Vaccination with TriMix and HTI mRNA is confirmed to be safe

A total of 33 participants were enrolled in the phase 2a of the clinical trial with respectively 8, 9 and 16 participants in the Placebo (physiological solution), TriMix (300µg TriMix) and HIVARNA (300µg TriMix and 900µg HTI) arm. Characteristics of participants did not differ between groups and are given in table 6.

Table 6. Characteristics of all phase 2a participants

n = 33	**Placebo (n = 8)**	**TriMix (n = 9)**	**HTI + TriMix (n = 16)**
Median age (IQR)	40.0 (35.0 - 54.0)	46.0 (35.0 - 54.0)	47.5 (36.5 - 54.0)
Male (%)	8 (100%)	9 (100%)	15 (94%)
MSM (%)	7 (88%)	8 (89%)	14 (88%)
Heterosexual	1 (12%)	0 (0%)	1 (6%)
Intravenous drug user	0 (0%)	1 (11%)	0 (0%)
Median CD4+ T cell count at			
- baseline (IQR)	771 (682 - 868)	771 (624 - 926)	792 (664 - 950)
- week 6 (IQR)	760 (624 - 1130)	771 (624 - 926)	775 (632 - 942)
- cART restart (IQR)	668 (556 - 770)	770 (622 - 921)	751 (595 - 875)
- end of study (IQR)	771 (640 - 919)	770 (622 - 921)*	775 (639 - 920)

Characteristics of phase 2a study participants, grouped by treatment arm. Group differences were tested using Kruskal-Wallis H test. * results of one patient omitted (did not restart ART).

All 33 patients received all three inguinal intranodal injections. Two weeks after the last injection, one patient developed a serious AE, not related to the vaccination and dropped out of the study. The remaining 32 patients interrupted cART therapy per protocol. Confirming the results from phase I, administration of Trimix and/or HTI mRNA was generally safe as no

grade 3 or above AEs were recorded that were related to the vaccination. Over the entire follow-up period, all patients experienced at least one AE, with a total of 206 AEs recorded. The total number of AEs was not significantly different between the three intervention groups.

Interim analysis

The interim analysis was performed after 33 patients completed their week 6 visits. The results of statistical testing dictated to stop any further inclusions due to futility.

Vaccination with Trimix and HTI does not delay viral rebound

All participants experienced viral rebound within two months post ATI (Fig. 7). Rebound peak VL values ranged from $3x10^3$ to 10^7 cps/mL but no significant differences were observed between study arms. The majority of patients (20 out of 32) restarted cART before week 18, on advice of the treating physician. Ten patients restarted cART at week 18 of the study and one patient at week 24, because of a very low plasma viremia at week 18. One patient did not restart cART, as he was lost to follow-up. Of the 31 participants that restarted treatment, 24 suppressed viremia again <50 cps/mL after 12 weeks, the remaining seven suppressed viremia later, which was secured during routine follow-up visits. No differences in timing of suppression were observed between study arms.

HIVARNA vaccination does not induce vaccine-specific T cell responses

Vaccine-specific T cell responses were assessed by peptide ELISpot and VIA. The ELISpot used peptide pools covering the HTI immunogen sequence (IN) as well as pools covering the rest of the HIV proteome not included in the HTI sequence (OUT). The VIA only used a pool of all HTI peptides.

In contrast to the moderate increases observed in phase 1, no significant increases were observed in number of T-cell responses against 'IN' peptides between baseline and week 6

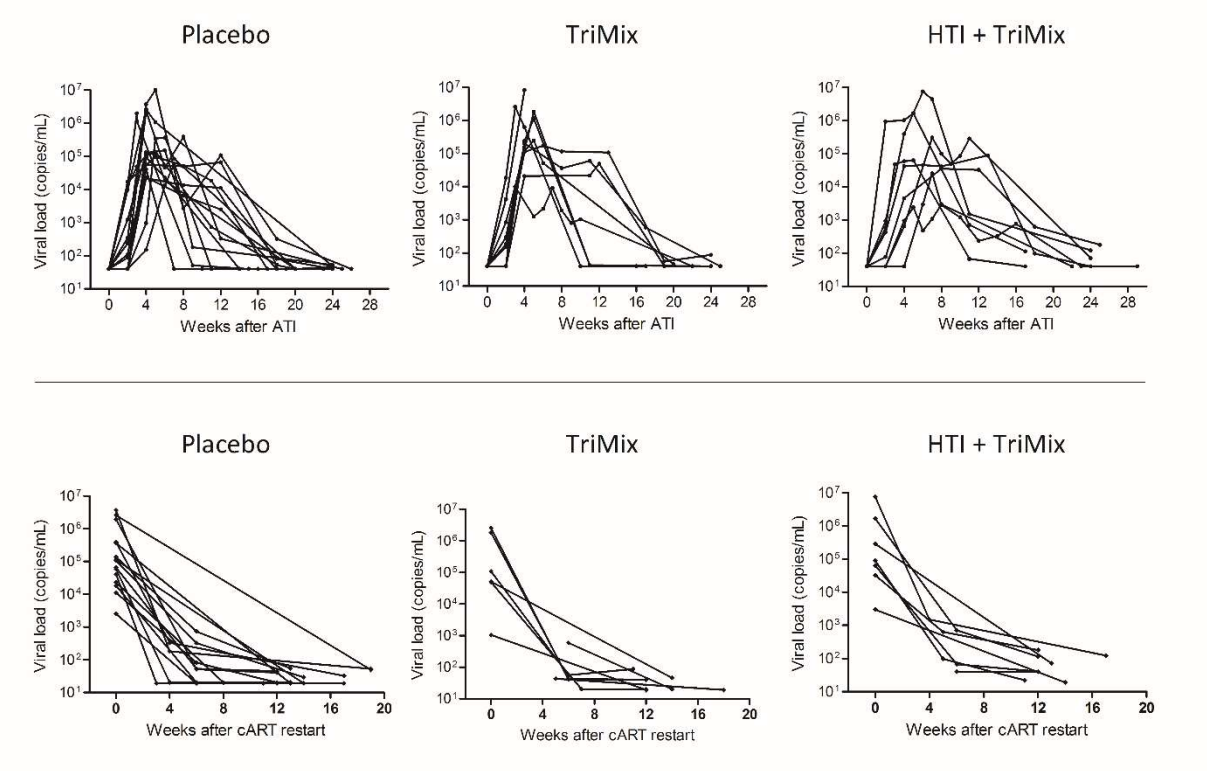


Figure 7: Plasma viral load shown relative to the start of treatment interruption to End of Study (EOS, top row) and relative to the stop of treatment interruption to EOS (Bottom row) in Placebo (n=8), TriMix (n=9) and HTI + TriMix (n=15) group (including no restart of ART for n=1). Results for subject ISC-402 are omitted (no treatment interruption due to SAE). (cART = combination antiretroviral therapy)

(Fig. 8). No increases in 'OUT' responses were observed either. Based on this lack of T cell responses, further inclusion of patients was stopped due to futility. Nevertheless, increases to both 'IN' and 'OUT' peptides were observed after treatment interruption, although these were not significant. These increases were earlier and higher in patients receiving TriMix and HIVARNA as compared to the placebo group.

In addition, we decided post-hoc to perform the more sensitive VIA assay to more conclusively determine whether the vaccine was immunogenic or not. VIA was performed with unstimulated as well as stimulated CD8+ T cells. The former are the most representative of

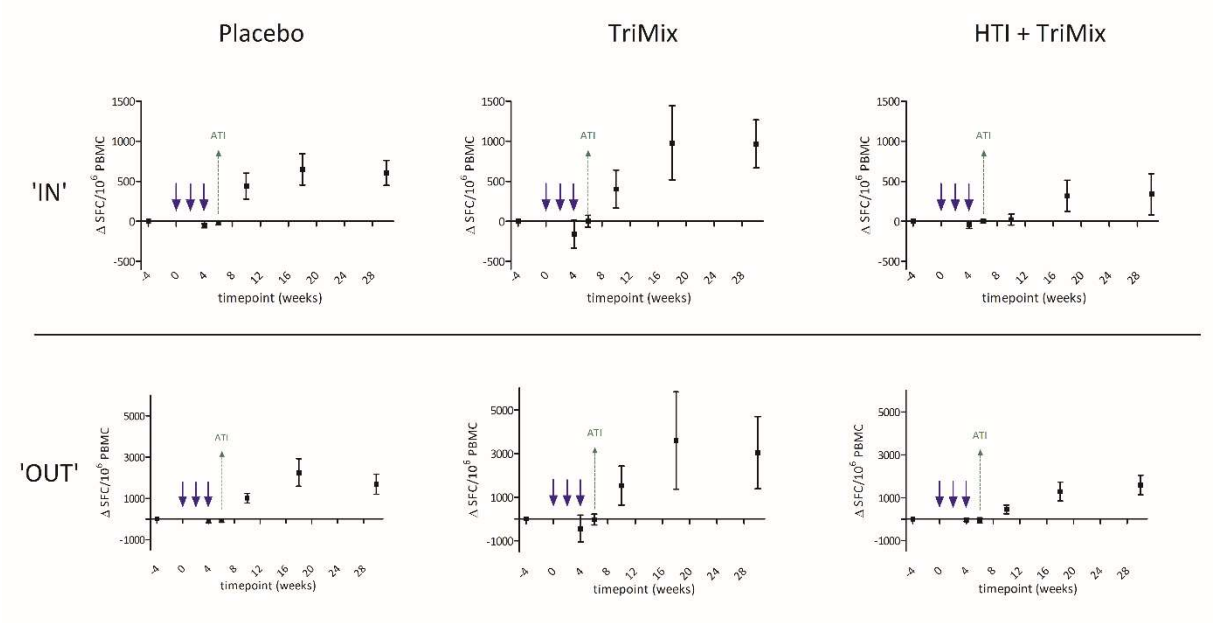


Figure 8. Primary efficacy endpoint: HIV-specific responses presented as change from baseline in either Placebo (n=8), TriMix (n=9) (including no restart of ART for n=1) or HTI + TriMix arms (n=15). Top row shows responses to HTI/IN peptide pools, bottom row shows responses to the remainder of the viral protein sequences (OUT peptide pools). Arrows indicate vaccinations. (SFC: spot forming cells; PBMC: peripheral blood mononuclear cells; ATI: analytical treatment interruption)

the ex-vivo situation while the latter have the advantage of being more sensitive, with typically much higher inhibition values than unstimulated cells [260]. We performed the assay with a high and low effector to target ratio (E:T), namely 2:1 and 0.1:1 respectively. Due to the study design and restrictions in the amount of blood that can be drawn per visit, it was not possible to assess VIA at the same time points (i.e. W-4, W0 and W6). Instead, we assessed VIA at baseline (W-4 and W0) and W4, just before the last vaccination.

We did not observe any significant change in VIA between baseline and week 4, at either E:T ratio, with unstimulated or stimulated CD8+ T cells (Fig. 9). The results presented here do not preclude, however, the possibility that VIA might have been increased by week 6.

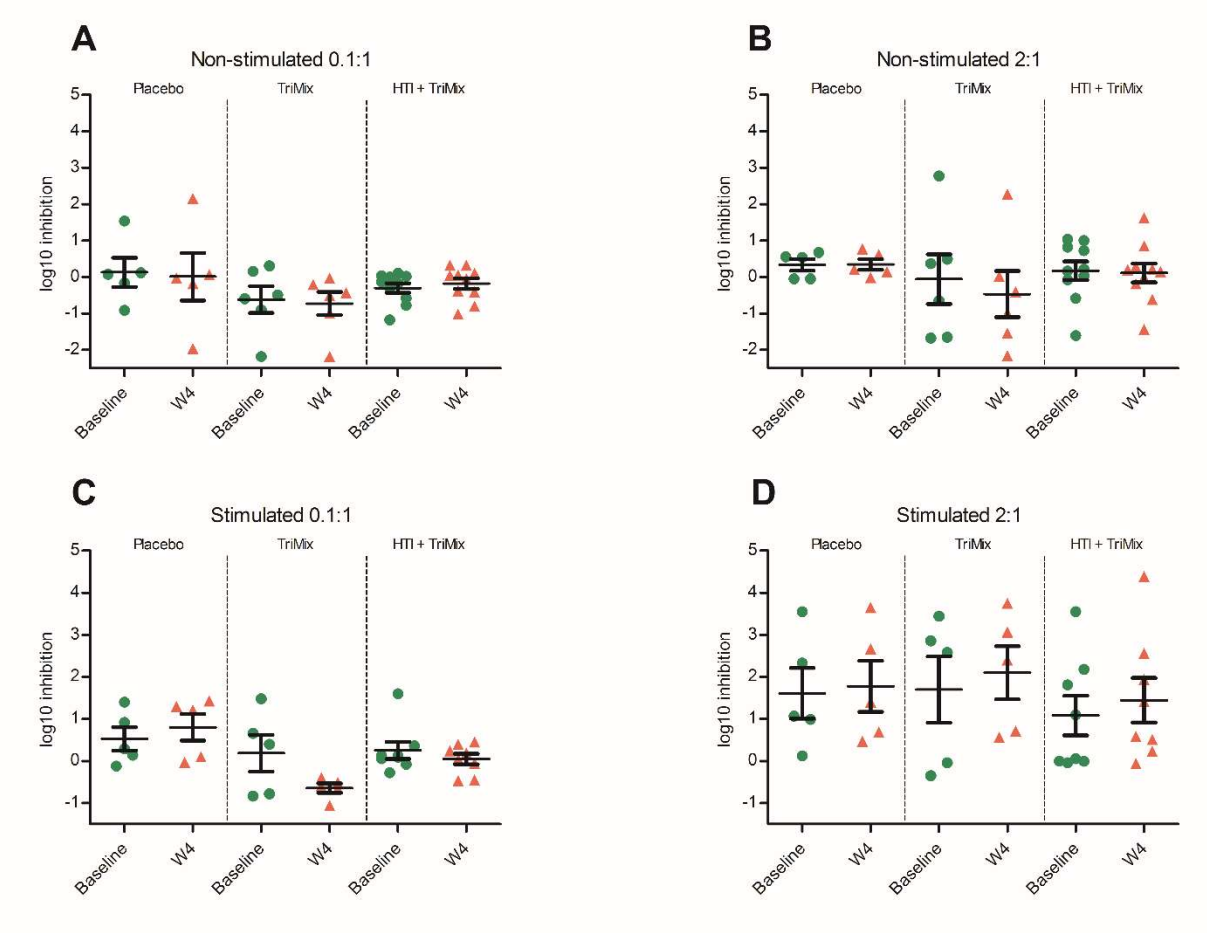


Figure 9. Viral inhibitory activity of CD8+ T cells in all three groups (placebo, TriMix and HTI TriMix) at baseline (average of W-4 and W0) and W4 without stimulation (A, B) and with stimulation (C, D) at effector to target ratios of 2:1 and 0.1:1. Mean with SEM is depicted.

Vaccination does not induce increases in viral DNA, cell-associated viral RNA or plasma viral load

No significant changes were observed at week 6, as compared to baseline, in terms of pro-viral DNA and, in contrast with phase 1 of the study, cell-associated US-RNA (Fig. 10). HIV-1 DNA and US-RNA levels increased after treatment interruption and normalized again by 24 weeks after treatment restart.

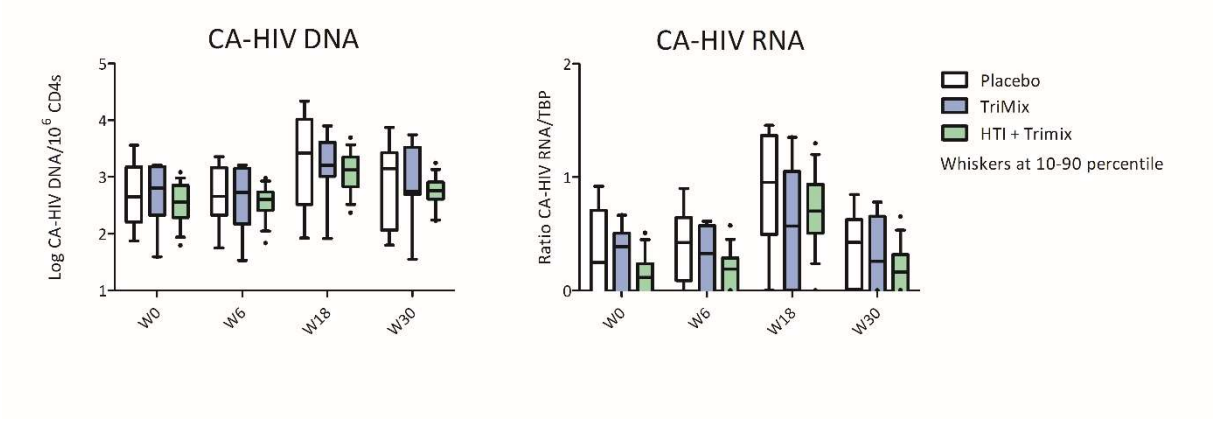


Figure 10. Levels of cell-associated (CA) HIV DNA (HIV copies per million CD4+ cells, left) and ratio of HIV RNA/TBP (housekeeping gene TATA box binding protein, right) in either Placebo (n=8), TriMix (n=9) (including no restart of antiretroviral therapy for n=1) or HTI + TriMix (n=15) group.

Finally, we assessed the effect of the vaccine on plasma VL by measuring usVL before and after vaccination. Due to the study design and restrictions in the amount of blood that was drawn per visit, it was not possible to obtain large volumes of plasma on week 6 (as in phase 1). Instead, usVL was determined on week 4, i.e. on the time point of the last vaccination. We did not observe an increase in usVL in the vaccinated group, nor in the placebo and TriMix control groups (Fig. 11).

Discussion

The iHIVARNA consortium was established to evaluate the feasibility, safety and immunogenicity of a novel naked mRNA vaccine strategy in humans in a phase 1 and phase 2a clinical trial. As explained in the introduction, the vaccination strategy was based on the combination of three innovations, namely an intranodal administration route proven to induce better immune responses as compared to the classical intradermal route in mouse models [255], an adjuvant composed of three different activation molecules which have been

shown to promote DC function *in vitro* and in mice [257, 258] and an immunogen corresponding to potentially protective target epitopes of HIV-1 specific CD8+ T cells [261]. In a preclinical

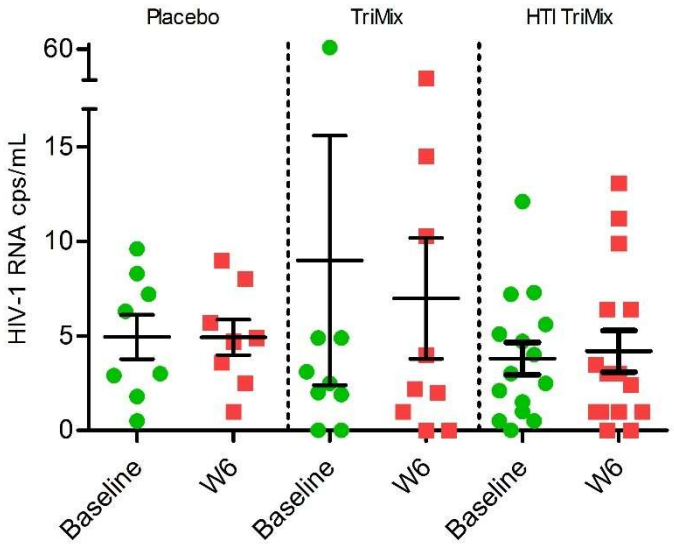


Figure 11. Ultrasensitive plasma viral load assay to quantify the effect of vaccination on HIV viral load below a threshold of <50 copies/mL. Data are presented as log_{10} HIV RNA copies/mL – Placebo (n=8), TriMix (n=9) (including no restart of ART for n=1), HTI-TriMix (n=15).

evaluation, this strategy already produced promising results, showing strong ex vivo activation of DCs in human lymph nodes and induction of HIV-specific T cell responses with *in vivo* lytic capacity against HTI antigen-loaded target cells in mice [256].

In the subsequent phase 1 and 2a clinical trials however, we were not able to confirm a definite immunogenicity of the vaccine. Due to the discovery of a serious error in the mRNA vaccine construct after completion of the second trial and its unknown impact on the expression of the vaccine, it is impossible to know whether this poor immunogenicity was intrinsic to the vaccine concept or due to poor translation of the mRNA caused by the error in the mRNA construct. The mRNA construct was found to contain an extra unintended start

codon upstream of the HTI open reading frame (ORF). This extra start codon gives rise to an alternative ORF that produces a 15 amino acid peptide. Importantly, this upstream ORF could have inhibited translation of the downstream ORF [262-264].

Consequently, it is conceivable that the HTI ORF has been expressed sub-optimally from the mRNA with a false upstream start codon. The intrinsic HIV-specific immunogenicity has only been observed in the preclinical evaluation where relatively much higher doses (of the exact same mRNA construct) have been used as compared to the clinical trials [190, 256]. Indeed, in the preclinical evaluation, mice were injected with approximately 3mg of total mRNA per kg bodyweight while participants of the human trials were vaccinated with about 13µg of total mRNA per kg bodyweight[a]. This higher dosage of more than two orders of magnitude in mice might have compensated the poor expression of the vaccine. Of note, safety and moderate immunogenicity of direct injection of naked mRNA have been demonstrated in previous clinical trials using dosages which are comparable to the dosage used in the iHIVARNA trial [265-267].

Interestingly, increases in T cell responses have been observed in both phase 1 (in the groups with the highest mRNA doses) and phase 2 of the study four weeks after treatment interruption (to both 'IN' and 'OUT' peptides). A possible explanation for this observation is the known adjuvant effect of using mRNA as a vaccine. In fact, any RNA can (non-specifically) stimulate the immune system through triggering of toll-like receptors, an effect which is not affected by the error in the vaccine construct. The effect of TriMix alone may also account for

[a] Assuming an average body weight of 17g for a female Balb/c mouse between six and 12 weeks old (https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/body-weight-chart-000651#) and an average bodyweight of 70kg for an adult human participant.

the observed immune stimulation. Alternatively, increases of HIV-specific T cell responses in phase 2 of the study might have been caused by stimulation of the immune system by the rebounding virus.

The vaccine mRNA itself was produced by one of the partners of the iHIVARNA consortium by *in vitro* synthesis of mRNA starting from a DNA plasmid containing the HTI ORF. This certified pharmaceutical grade plasmid DNA was in turn manufactured by an ISO 9001 certified third party. Several quality controls have been performed, including restriction enzyme digestion, concentration measurement by spectrophotometry, agarose gel electrophoresis to determine the percentage of covalently closed circular plasmid DNA, determination of the endotoxin content, verification of the presence of E. coli and confirmation of sterility. Nevertheless, it seems that the crucial quality control on sequence data of the plasmid, which would have exposed the upstream ORF, was not pursued. It is only after completion of both trials that such a sequence analysis was performed and that the anomaly was revealed. While a thorough investigation into this unfortunate incident is on-going, new standard operating procedures have been adopted by the partner responsible for the production of the mRNA, in order to avoid similar incidents in the future.

While we cannot make statements about the immunogenicity of our vaccination strategy due to this error, we fortunately did not observe any serious adverse events related to the vaccination, neither in phase 1 nor in phase 2 of the study. In this regard, it is noteworthy that the 15 amino acid long aberrant peptide resulting from the upstream ORF does not show any significant homology with any known human sequences (≤7 identical residues) but does show significant homology with bacterial proteins (up to 13 identical residues). In addition, all participants who interrupted therapy and completed the study returned to pre-ATI levels of

HIV-1 DNA and US-RNA as well as to undetectable plasma VL levels. Consequently, despite the psychological impact of participating in an ATI and the time investments of the study participants, it is safe to conclude that the impact on the participants' health has been limited.

In conclusion, we were unfortunately not able to evaluate the immunogenic potential of this promising, innovative vaccination strategy due to a serious error in the vaccine itself. Careful follow-up studies on the original concepts remain indicated.

Chapter 7 - General discussion, conclusion and perspectives

Achieving a functional cure of HIV

Over the past decade, there has been a major increase in interest from the scientific community into HIV curative strategies. An important factor in this surge of enthusiasm was the report in 2009 of the first man ever to be cured of HIV, the 'Berlin patient', after repeated whole body irradiation and hematopoietic stem cell transplantation (HSCT) with CCR5 Δ32/Δ32 stem cells [51]. Exactly ten years later, the 'London patient' who received a similar treatment and has been in remission for more than 18 months, might become the second man to be cured of HIV [52]. Although inspiring, such life-threatening treatments, including the destruction of the entire patients' own immune system, followed by HSCT, cannot be generalized in "healthy" HIV-infected subjects under effective and well-tolerated cART.

Alternative strategies to eradicate latent HIV reservoirs include gene therapy to eliminate proviruses from infected cells and the 'shock and kill' strategy where infected cells are reactivated first and eliminated by the immune system afterwards [268]. These eradication strategies have had very modest effects until now, however, and it has become increasingly clear that achieving a sterilizing cure, by completely purging the viral reservoir, will be very difficult. Consequently, the main focus of HIV cure research has shifted towards achieving a functional cure, i.e. an intervention to induce permanent suppression of HIV replication without the continuous need for drugs after a given period of combination antiretroviral therapy. **A consensus now exists that achieving such control after treatment interruption (TI) will require both immune control as well as reservoir size reduction.** Indeed, today, a number of studies, including our own, have shown that even patients with extremely small reservoirs are not more likely to achieve functional cure after ATI [53, 174, 204].

When this thesis was designed, however, the above consensus did not yet exist. As a result, the influence of immune control and viral reservoir size were studied separately. Namely, the ISALA trial investigated whether a small and transcriptionally silent viral reservoir makes "spontaneous" functional cure more likely. The iHIVARNA trial on the other hand, studied the immunogenicity of an innovative therapeutic vaccine and its capacity to delay viral rebound and potentially induce functional cure. The main goals and conclusions of this thesis are schematically summarized in fig. 1.

The ISALA study: rapid viral rebound despite small and transcriptionally silent viral reservoir

Previous studies have observed an association between delayed viral rebound and the size of the viral reservoir, measured as HIV-1 proviral DNA levels (t-DNA) [179, 192, 269]. Consistent with these findings, exceptional patients who were treated from the early phase of infection, but also some who were treated during the chronic phase, were found to achieve viral control after TI, presumably because of their small viral reservoir [54, 187, 270]. On the other hand, an association was also observed between delayed viral rebound and the transcriptional activity of the viral reservoir, measured as HIV-1 cell-associated unspliced RNA levels (US-RNA) [181, 192].

Based on these separate associations, the objective of the ISALA TI study was to determine whether the combination of a very small size and minimal transcriptional activity of the viral reservoir was predictive of delayed viral rebound (**chapter 3**). Patients with t-DNA and US-RNA levels below a chosen threshold were included in the study and underwent a closely monitored TI. Disappointingly however, rapid viral rebound after TI was observed in all participants. It is important to note, however, that very few patients were excluded from participation based on the transcriptional activity of their viral reservoir (US-RNA), because

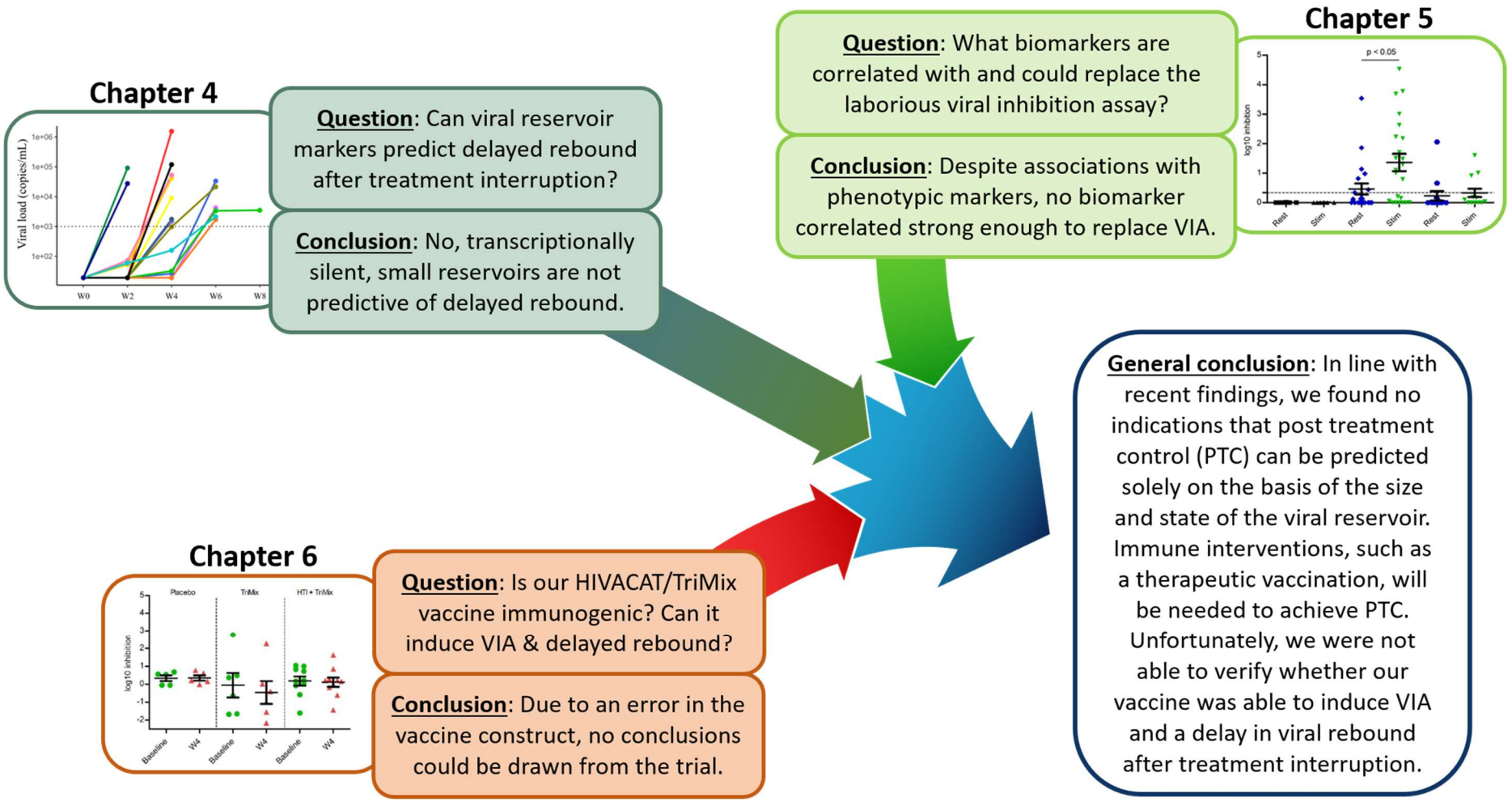


Figure 1. Schematic summary of the main goals and conclusions of this thesis work.

most patients with small t-DNA levels had undetectable US-RNA levels anyway. As a result, participants were mostly selected based on t-DNA levels, making it very similar to the studies which only focused on the size of the reservoir and equally failed to identify post-treatment controllers [204].

In retrospect however, considering (i) the higher sensitivity of t-DNA as compared to US-RNA detection and (ii) the strong correlation between t-DNA and US-RNA levels in patients on cART, this is not entirely surprising and perhaps should have been taken into account during the design of the trial [228]. US-RNA is a conceptually relevant biomarker, as it is the result of late-stage transcription events and therefore likely associated with actual production of new virions. But markers of early-stage transcription events, such as LTR-RNA, are up to 100 fold more abundant and are therefore much more sensitive indicators as compared to US-RNA [197]. Retrospective studies should determine whether these indicators of early transcription are associated with delayed viral rebound. If this is the case, future prospective trials studying the importance of transcriptional activity should consider including markers of both early as well as late transcription.

While the overall low US-RNA levels precluded a strong selection based on transcriptional activity, this was not the case for t-DNA levels. Nevertheless, patients with very small amounts of t-DNA were not more likely to control viremia after TI than the general HIV+ population on cART. On the one hand this suggests that an additional immune intervention is needed to achieve viral remission, on the other hand this reaffirms the idea that measuring the level of t-DNA with PCR technology is not an appropriate way of assessing the size of the latent reservoir. Indeed, extensive whole genome sequencing has shown that the majority of all proviruses are defective as they contain large internal deletions and hypermutations, and

cannot lead to new productive infection anymore [33]. Only a small, variable percentage of proviruses are intact and can potentially be reactivated to produce new infectious virions [35].

This proportion of intact and reactivatable proviruses (the replication competent fraction, RCF) can be estimated with a quantitative viral outgrowth assay (qVOA), but unfortunately, this biomarker is known to largely underestimate the RCF [34]. In addition, qVOA requires a large number of cells and can therefore only be performed on leukapheresis samples. As a result, qVOA is not suited to be used prospectively as a screening tool. In the ISALA trial, all 16 participants who interrupted treatment were retrospectively tested with the qVOA. No correlation was observed between the qVOA and the TTVR. Considering the very small differences in TTVR in between patients and their very similar qVOA results, this lack of correlation is perhaps not surprising.

A more practical and perhaps more relevant way of measuring the latent reservoir might be a combination of measuring the amount of proviruses (t-DNA as measured in the ISALA study) and determining what proportion of those are intact with full genome sequencing. In doing so, an absolute amount of intact proviruses could be determined and a more accurate and relevant size of the latent reservoir could be obtained.

Probably the most important limitation of this study is that all reservoir measures (t-DNA, integrated DNA, US-RNA, LTR-RNA, qVOA) were determined in cells from the periphery, which probably represent only 1% of infected cells, the remaining 99% being present in the numerous tissue reservoirs located throughout the body [58, 59]. It is as yet unclear how representative the periphery is for the entire reservoir. It has been shown, however, that concentrations of infected cells are much lower in peripheral blood as compared to certain tissues, which inherently decreases the sensitivity of our viral reservoir measures. On the

other hand, tissues are hard to sample in a representative way and obtaining tissues samples is also much more invasive than drawing blood. As a result, most studies still focus on peripheral blood.

In conclusion, this study supported **the consensus that functional cure is not simply the result of a sufficiently small viral reservoir and that the extra factor of immune control is probably required** to prevent viral rebound after TI.

Assessing CD8+ T cell mediated immune control of viral replication

The disappointing results from the ISALA study made it clear that an immune intervention is needed to achieve sustained viral control after TI. However, there is to date no ideal assay to measure the efficacy of such an immune intervention and hence, of any clinical trial aiming to induce HIV-specific, protective CD8+ T cell responses. Traditional assays such as interferon-γ ELISpot and intra-cellular cytokine staining indirectly measure CD8+ T cell function and have proven to be of limited value in terms of predicting *in vivo* viral control [101, 102]. In contrast, the viral inhibition assay (VIA) directly measures the capacity of CD8+ T cells to suppress viral replication and is considered one of the most physiological ways of testing antiviral activity (**chapter 2**). In preparation of the therapeutic vaccination trial (iHIVARNA, **chapter 5**) we conducted a cross-sectional study with patients on cART with undetectable viral loads, to (i) set up an in-house VIA assay to be used in the iHIVARNA trial and (ii) further explore possible correlations with clinical, viral and immune parameters (**chapter 4)**.

We established a reproducible VIA assay with low background, because non-specific stimuli (such as interleukin-2) were avoided during the preparation of the effector CD8+ T cells. As expected, VIA activity of unstimulated ex vivo CD8+ T cells from cART patients was limited, but *in vitro* prestimulation with an HIV gag peptide pool significantly boosted their viral

suppressive capacity. We hypothesize that during this stimulation, antigen presenting cells process the HIV peptides and present them to the CD8+ T cells, thereby causing an expansion of existing HIV-specific memory CD8+ T cells and a reversal to a more functional phenotype. On the one hand, we show that we can measure precisely what immune interventions such as a therapeutic vaccination aim to induce, i.e. stimulation and expansion of HIV-specific memory CD8+ T cells that are able to inhibit viral replication. On the other hand, we set up **a VIA assay which is both specific (no background) and sensitive (higher VIA than non-stimulated version), resulting in a promising tool to evaluate the efficacy of immune interventions**.

In our phenotypic analyses, we found that stimulated VIA was significantly correlated with higher co-expression of classical immune exhaustion markers PD1 and CD160 on subsets of terminally differentiated effector memory CD8+ T cells (so called TEMRA cells). We also observed higher expression of CD57 on effector memory (EM) and TEMRA cells, a marker of terminal differentiation, anergy and senescence. Finally, we observed higher HLA-DR expression on CD8+ T cells during co-culture in suppressors, thus confirming the previously observed association of HLA-DR with VIA [112].

It is somewhat surprising to observe associations of VIA with markers of exhaustion, terminal differentiation and senescence. Therefore, our findings might contribute to a revision of the biological significance of these markers. Indeed, besides immune exhaustion, PD1 is first of all a marker of T cell activation and is even related to the strength of T cell receptor signaling and thus with functional avidity [271-273]. In turn, co-expression of CD160 and 2B4 (both also considered as immune checkpoint markers) has been correlated to a functional, cytolytic subset of CD8+ T cells in elite controllers [274]. The association of CD57 with T cell anergy is

unclear [275] and similarly, senescence does not equate to exhaustion, but rather corresponds to a reduced proliferative capacity with still a strong potential of cytokine secretion [247, 276]. Taken together, the expression of PD1/CD160, CD57 and HLA-DR on specific subsets of CD8+ T cells of suppressors suggests that these highly activated and terminally differentiated cells can still be highly functional in their capacity to inhibit viral replication, despite a possibly reduced proliferation capacity. It also warrants caution when using terms such as senescence and exhaustion since they are not synonyms of "not functional at all".

In an editorial comment on our paper, Zaunders *et al.* elaborated on this apparent contradiction between better CD8+ T cell functionality and higher expression of immune exhaustion markers. Since we did not observe strong associations between VIA and classical cytotoxicity markers, they suggest that the viral inhibition we observed might be explained by cytokine production rather than through direct cytolytic mechanisms. They then go on to hypothesize that immune checkpoint inhibitors such as PD1 might indeed restrict cytotoxic activity and proliferation, as is generally assumed, but leave cytokine production unaffected. To verify this hypothesis, further studies are needed where CD8+ T cells expressing these immune checkpoint markers are isolated and tested for their cytotoxicity versus their noncytolytic antiviral suppressive activity.

The iHIVARNA study: therapeutic vaccination to induce immune control after TI

While the ISALA study focused on the importance of a small reservoir for functional cure (**chapter 3**), the iHIVARNA study aimed at inducing protective immune responses by means of a therapeutic vaccination strategy (**chapter 5**).

The iHIVARNA trial evaluated the safety (phase 1) and immunogenicity (phase 2) of an innovative vaccination strategy, based on direct intra-nodal injection of naked mRNA coding

for a set of conserved HIV peptides and three DC maturation stimuli. In this case, study participants were not selected based on small reservoir size and/or transcriptional activity (as in the ISALA study), or having started treatment in the acute phase of the infection.

While no serious adverse events were observed, no significant increases in vaccine specific T cell responses after vaccination were observed either, as assessed by IFN-γ ELISpot and VIA. As discussed in **chapter 5** however, the vaccine was found to contain a serious error, causing doubts on whether the vaccine mRNA was efficiently translated or not. As a result, no conclusions could be drawn about the safety or the immunogenicity of the vaccine.

More precisely, an extra start codon was found upstream of the intended HTI start codon, resulting in an alternative upstream open reading frame (uORF). Interestingly, more than 40% of human genes contain such uORFs in their transcript leader regions and are recognized to play a role in the regulation of mRNA translation through mechanisms which are not yet fully understood [277]. After a ribosome has recognized and translated an uORF, it can remain associated to the mRNA and reinitiate translation further downstream [278, 279]. Whether this happens or not and with what efficiency, depends on the secondary structure of the mRNA, amongst other factors [280]. Alternatively, the ribosome can stall during either the elongation or termination phase of the uORF translation, thereby blocking additional scanning ribosomes and effectively inhibiting translation of the ORF of interest. At the time of writing, this kind of structural information of the mRNA construct is not available and it is therefore impossible to estimate whether translation of the HTI ORF was indeed significantly inhibited or not.

In order to try and valorize the iHIVARNA trial data, further in silico and *in vitro* investigations into the translation efficiency of the faulty mRNA vaccine are warranted. The results from these investigations will inform us on (i) whether the vaccine was never translated, in which

case we have to accept that the trial data are invalid, or (ii) whether the vaccine was efficiently translated despite the uORF, in which case a likely conclusion of the study would be that the vaccine was under dosed. In any case, the "negative results" of the iHIVARNA trial do not invalidate the concept of the carefully designed HIVACAT antigen, which is being evaluated in other vaccine formats (e.g. DNA prime and viral vector boost).

Therapeutic vaccination: past and future

Numerous previous vaccination trials have mostly used viral vectors and dendritic cell based vaccines [281, 282]. The iHIVARNA study was the first in-human trial to use naked mRNA as a therapeutic vaccine for HIV. Previously, the use of mRNA has been restricted by its instability, poor *in vivo* delivery and inefficient translation. Recently however, several optimizations (including better stability through modification of the 5' leader region, more efficient translation by improved codon usage, modified antigen-processing characteristics of the encoded protein, combination with potent adjuvants, etc.) have turned mRNA into potent gene vaccination vehicles.

While some therapeutic vaccines aim to redirect host responses to a number of the most conserved HIV peptides (as HIVARNA's HTI immunogen) [281, 283, 284], other more recent trials aim to broaden the immune response to recognize as much escape variants as possible (mosaic vaccine) [82, 117, 282, 285]. The most promising vaccine trials to date are those eliciting broad rather than narrow immune responses, having observed the strongest effect on (set-point) viral load [282, 286] and delay in viral rebound after TI [287, 288]. In any case, none of these randomized controlled trials has been able to induce long-term remission after TI [289] and it is likely that vaccine trials have been unsuccessful at inducing durable viral control after ATI exactly because the elicited immune responses were not able to recognize the diverse

rebound viruses (i.e. not broad enough). Nevertheless, there is hope, as significant success has been seen with therapeutic vaccines in SIV-infected non-human primates, an animal model for HIV infection in humans.

As an example, one study in SIV-infected monkeys on cART combined a therapeutic vaccine, consisting of two viral vectors expressing Gag, Pol and Env, with a toll-like receptor 7 (TLR7) agonist. Triggering TLR7 activates dendritic cells and lymphocytes and leads to innate immune activation with secretion of cytokines and chemokines as a result [290, 291]. The magnitude and breadth of Gag/Pol/Env responses were strongly increased in response to the vaccination, with several animals developing T cells targeting more than 50 epitopes. A striking reduction in set-point VL and delay in viral rebound were observed after TI. One third of the monkeys even remained undetectable after TI. Importantly, the breadth of the cellular immune response correlated inversely with set-point viral load and directly with delay in viral rebound [292]. Similar in-human studies are being planned.

A cure for HIV: still a priority today?

Besides finding a cure, other priorities exist today in the fight against the HIV pandemic. Decreasing the number of new infections, increasing the number of new diagnoses and increasing access to quality care are arguably more pressing issues to tackle today, as will be discussed later in this chapter. Considering how well-tolerated and effective current antiretrovirals are at suppressing viral replication, the question whether a cure for HIV should be a priority should be asked.

The answer to this question however, lies in the fact that people living with HIV need to take their antiretroviral drugs every single day for the rest of their lives. This is not only costly but may still have a long-term impact on the patients' health. In addition, by continuing to take

drugs every day patients are each time reminded that they are infected by a virus for which they are stigmatized. A cure for HIV would help removing this stigma and might also encourage people to get tested since a positive diagnosis would not equal a life-long sentence anymore. Finally, a cure is also likely to be the most, if not the only, way of halting transmission and ultimately solving the HIV problem. Taken together, HIV cure definitely remains an important research priority, but will we ever find one?

Although many challenges remain, the wide variety of promising areas that are being investigated besides therapeutic vaccination, gives hope that a durable and scalable cure will one day be possible [177]. A lot of work is being done on characterizing and quantifying the latent viral reservoir, providing ever more useful insight into the meaning of latency, how to reverse it and what parts of the reservoir to target [37]. More potent and specific latency reversing agents (LRAs) are being developed to reactivate the latent reservoir [71] and the inhibition of immune checkpoint markers is being investigated to reverse immune exhaustion and improve the capacity of CD8+ T cells to eliminate reactivated, infected CD4+ T cells [293]. An innovative way of mediating very specific cytotoxic T lymphocyte responses utilizes engineered T cells (CAR T cells) which have been modified to express an artificial T cell receptor and recognize specific HIV peptides [294]. Another way of inducing CD8+ T cell responses with the use of broadly neutralizing antibodies is being investigated as well [295]. All of the above interventions are part of the 'shock and kill' strategy, aiming to purge the reservoir by means of cytotoxic T cell responses after having reactivated the latent reservoir. As opposed to reactivation, the relatively new 'block and lock strategy' aims at inducing a state of 'deep latency' by blocking transcription with the use of a *tat* inhibitor [72]. Another strategy that is being investigated to reduce the size of the replication competent reservoir is gene therapy,

which includes methods of inactivating proviruses by excising integrated proviral genomes from latently infected cells with engineered nucleases [296].

More likely than not, a functional cure will be the result of a combination of several of the above strategies. Such a "complete" approach to curing HIV could consist of an initial phase of reducing the size of the reservoir under the protection of cART, using gene therapy and/or engineered T cells to inactivate proviruses and eliminate infected cells respectively. Next, an effective therapeutic vaccination would elicit an abundance of HIV-specific cytotoxic T cells, targeting a broad range of possible escape variants, after which potent LRAs could be administered to reactivate the remaining latent reservoir and make infected cells visible targets for elimination by the vaccine induced cytotoxic T cells. The vaccination could be combined with the administration of antibodies targeting immune checkpoint inhibitors, in order to rejuvenate the immune system and increase the effectiveness of the vaccine. Finally, a block and lock component could be added to completely silence whatever intact provirus still remains.

HIV cure, what else?

Nevertheless, an actual HIV cure is still many years away and low and middle income countries (LMIC), which carry the highest burden of the disease, will likely get access to such a cure even later. Today, around 20 million people living with HIV still do not have access to cART. Consequently, they have unsuppressed viremia, are at high risk of transmitting the virus and are not protected against progression of the disease. As a result, nearly 2 million people get newly infected and nearly 1 million people die of AIDS related illnesses each year. This is a public health emergency and should be responded to as such, even though it has been an emergency for many years already. While finding a cure is of utmost importance on the long

term, it clearly is not part of an emergency response. The current top priorities of the global response against HIV/AIDS should therefore be focused on finding HIV positive people, getting them on treatment and making sure they maintain undetectable viral loads. This not only benefits the people themselves by halting disease progression, but also avoids new infections, as undetectable viral loads are virtually untransmittable, whence the name of the UNAIDS explainer “U = U” (undetectable = untransmittable) [297-300].

As an example of an important aspect of the fight against HIV/AIDS which deserves more money and research focus, let us look at viral load (VL) monitoring. Regular VL testing is a crucial part of patient follow-up after treatment initiation, to monitor treatment adherence of the patient and development of resistance mutations of the virus. Many people on treatment still have no access to viral load monitoring and several studies show that viral load results do not always lead to prompt and appropriate clinical action. Viral failure (defined in LMIC as a VL >1000cps/mL by the World Health Organization) is often not followed by adherence counseling [301] or a follow-up viral load test [302]. Other studies have reported result turnaround times (TAT) of 30 weeks and time to treatment switch after confirmed viral failure of 68 weeks [303]. While part of these programmatic failures are symptomatic of weak health systems, new technologies could help solve some of these problems. Point-of-care viral load testing has the potential of increasing access and dramatically decreasing result TAT, allowing prompt clinical action after confirming viral failure. eHealth, i.e. using information and communication technology in health systems, can also reduce result TAT as well as reduce the number of patients lost to follow up. More research and development funding should go to these types of technology that allow more efficient follow-up of patients on cART and help these patients achieving and maintaining an undetectable VL.

Finally, I believe it is appropriate to end this discussion section of my thesis on a positive note. Namely, the field of long-acting and extended release (LA/ER) formulations of antiretrovirals has made some very promising progress to the extent that it might cause an actual paradigm shift in how we look at cure, treatment and even prevention of HIV altogether. LA/ER formulations include antiretroviral drugs which are injected intramuscularly (as a drug depot) or subcutaneously (as a nano-implant) and are slowly released, but also include oral drugs formulated as solid drug nanoparticles and injections of broadly neutralizing antibodies with (very) long half-life. In this way, they offer protection over an extended period of time, thereby replacing the need for daily oral medication [304, 305].

The current pipeline of LA/ER formulations consists of oral products providing protection for several weeks after a single dose, to implants providing protection for several months. At the latest Conference on Retroviruses and Opportunistic Infections (2019), preliminary results were presented of the on-going FLAIR study (First Long-Acting Injectable Regimen), a large multi-centric, randomized, open-label, non-inferiority study comparing the effectiveness of maintaining suppressed viral load with monthly intramuscular injections (cabotegravir/ rilpivirine) versus daily oral medication (dolutegravir/abacavir/lamivudine). At week 48, the monthly injections were observed to be non-inferior to the oral medication and, also importantly, were the preferred treatment mode for more than 90% of the participants [306].

Continued research efforts will undoubtedly allow even less frequent injection schemes, adapting currently existing anti-retrovirals and monoclonal antibodies to reach longer half-lives. Nevertheless, research will also have to solve a number of related problems and challenges. These include the coverage of the long tail of drug concentrations, pregnancy, appropriate dosage for infants and children, irreversible or long-lived side effects, drug-drug

interactions, the injection volume which is relatively large and the need for an oral lead-in, amongst others [305, 307].

Once these challenges are overcome however, the advantages of LA/ER formulations are potentially game-changing. They are ideal to tackle the problem of poor treatment adherence, an important cause of viral failure and drug resistance development, which is taking on large proportions in LMIC [308, 309]. They are also a solution to pill fatigue and a convenient alternative in places where stigma of daily pill intake is a problem. The concept of LA/ER is ideal for infants and children where daily drug intake is challenging. The enormous logistical challenges related to ensuring an uninterrupted drug supply would also be strongly simplified in hard to reach places of LMIC [310]. In addition, as long as it does not become self-administered, it effectively becomes a directly observed treatment, further ensuring drug faithfulness. Surveys in sub-Saharan African countries have observed rates of anti-conception implants of more than 50% among women using any anti-conception method, indicating that it already is a well-accepted delivery method [311]. Moreover, thanks to their favorable pharmacokinetics, solid drug nanoparticles have the potential of decreasing drug costs by up to 50% [312, 313].

LA/ER formulations are also ideally suited for pre-exposure prophylaxis strategies, thereby becoming a long-lasting disease prevention method and almost starting to resemble an immunization. Obviously, the possibilities go far beyond HIV as LA/ER strategies would be equally well-suited for diseases like tuberculosis, hepatitis B and C, malaria or even Ebola [314, 315]. It is in this perspective that LA/ER formulations may well lead to a major paradigm shift in prevention and treatment of various infectious diseases in LMIC. In terms of prophylaxis, it has the potential of becoming an attractive alternative for vaccination of diseases for which vaccines are proving (very) difficult to develop. In terms of treatment, a long acting implant

providing protection of one or more years without causing side effects would immensely minimize the impact of a chronic disease on an individual's quality of life. Considering this care-free, disease-free and "pill-taking-free" state of the patient, LA/ER formulations would become worthy alternatives for an actual functional cure.

In conclusion, in a field as complex as the HIV/AIDS pandemic, there cannot be one single top priority. The pandemic consists of several epidemics which differ strongly depending on their geographical location. That being said, it is mostly in the LMIC where people are still dying from AIDS, where babies are being born with the virus, where many people are getting newly infected every year. HIV cure research should therefore have its place on today's research agenda, but research which helps getting the remaining 50% of infected people on treatment and virally suppressed should be equally, if not more, prioritized.

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List of abbreviations

AE	adverse event
AIDS	Acquired immune deficiency syndrome
APC	antigen presenting cell
ARV	anti-retroviral
ATI	analytical treatment interruption
bNAB	broadly neutralizing antibody
CAR	chimeric antigen receptor
cART	combination anti-retroviral therapy
caTLR4	constitutively active variant of Toll-like receptor 4
CCR5	C-C chemokine receptor type 5
CCR7	C-C chemokine receptor type 7
CD	cluster of differentiation
cDNA	complementary DNA
CM	central memory
CMV	cytomegalovirus
CP	chronic progressor
CPS	clonal prediction score
CRA	chromium release assay
CTL	cytotoxic T lymphocytes
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CXCR4	C-X-C chemokine receptor type 4
DC	dendritic cell
ddPCR	droplet digital polymerase chain reaction
DLT	dose limiting toxicity
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid

E:T	effector to target ratio
EBV	Eppstein Barr virus
EC	elite controller
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ELISpot	enzyme linked immunosorbent spot assay
EM	effector memory
EMC	Erasmus Medical Center, Rotterdam
ENV	envelope
EOMES	eomesodermin
FBS	fetal bovine serum
FCS	fetal calf serum
FI	fusion inhibitor
FITC	fluorescein isothiocyanate
Gag	group-specific antigen
Gp	glycoprotein
GrB	granzyme B
GTR	general time-reversible
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HSCT	hematopoietic stem cell transplantation
HTI	HIVACAT T cell immunogen
IC	immune checkpoint
ICS	intracellular cytokine staining
IDIBAPS	Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona
IFN	interferon

II	integrase inhibitor
IL	interleukin
IN	integrase
INSTI	integrase strand transfer inhibitor
Iono	ionomycin
IQR	inter-quartile range
IRSICAIXA	Institut de Recerca de la Sida, Badalona
ITM	Institute of Tropical Medicine, Antwerp
IUPM	infectious units per million cells
IVD	intra-venous drug
kB	kilo base
LA/ER	long-acting and extended release
LAG3	lymphocyte-activation gene 3
LMIC	low and middle income countries
LOD	limit of detection
LRA	latency reversal agent
LTFU	lost to follow-up
LTNP	long term non progressors
LTR	long terminal repeat
LTR-RNA	long-terminal repeat cell-associated HIV-1 RNA
MA	matrix
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MIP	macrophage inflammatory proteins
Mo-DC	monocyte-derived dendritic cell
MOI	multiplicity of infection
mRNA	messenger rna
MSM	men who have sex with men

NA	nucleocapsid
NEF	negative factor
NIH	National Institute of Health
NK	natural killer
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD1	programmed cell-death 1
PE	phycoerythrin
PHA	phytohemagglutinin
PI	protease inhibitor
PMA	phorbol-12-myristate-13-acetate
Pol	polymerase
PR	protease
PTC	post-treatment control
pVL	plasma viral load
PW12	week 12 post-cART restart
qRT-PCR	quantitative real-time polymerase chain reaction
qVOA	quantitative viral outgrowth assay
RC	replication competent
RCF	replication competent fraction
Rev	regulator of viral protein expression
RNA	ribonucleic acid

RPMI	Roswell Park Memorial Institute
RPP30	Ribonuclease P protein subunit p30
RT	reverse transcriptase
SD	standard deviation
SDF-1	stromal cell-derived factor 1
SFC	spot forming cell
SGA	single genome analysis
SIV	simian immunodeficiency virus
SPADE	Spanning tree progression analysis of density-normalized events
SPARTAC	Short Pulse Anti-Retroviral Therapy at Seroconversion
Tat	trans-activator of trancription
TAT	turnaround time
T-bet	T-box transcription factor
TBP	TATA-binding protein
t-DNA	Total cell-associated HIV-1 DNA
TEM	effector memory T cell
TEMRA	terminally differentiated effector memory RA positive T cells
Th1	T-helper type 1
Th17	T-helper type 17
Th2	T-helper type 2
TI	treatment interruption
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TILDA	tat/rev induced limiting dilution assay
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TNF-α	tumor necrosis factor alpha
TTVR	time to HIV-1 viral rebound
U	unit
UMC	University Medical Center St Pierre

uORF	upstream open reading frame
usRNA	unspliced cell-associated HIV-1 RNA
US-RNA	unspliced cell-associated HIV-1 RNA
usVL	ultra-sensitive plasma viral load
UZB	Universitair Ziekenhuis Brussel
UZG	Universitair Ziekenhuis Gent
VC	viremic controller
VIA	viral inhibition assay or viral inhibitory activity
Vif	virion infectivity factor
VL	viral load
Vpr	viral protein r
Vpu	viral protein u
VRA	Viral release assay
VSC	viral suppressive capacity
VUB	Vrije Universiteit Brussel, Brussels
WFI	Water for injection
WHO	World Health Organization

Summary

Although combination antiretroviral therapy (cART) is very effective at suppressing viral replication and preventing disease progression, it is not curative due to the existence of a latent viral reservoir in resting memory CD4+ T cells. As a result, HIV infected patients need to take life-long antiretroviral therapy. Besides the high economic cost, it is proving to be very challenging to get all HIV positive people on treatment, especially in low -and middle income countries. In addition, although the pill burden has been reduced to one pill per day, daily treatment continues to affect quality of life and may still have a long-term impact on the patients' health. Therefore, a cure for HIV is needed.

It is becoming increasingly clear that achieving a 'sterilizing cure', where a patient becomes completely virus-free, will be very difficult. In contrast, achieving a 'functional cure', where a patient is still infected but suppresses the virus without the need for drugs after a period of therapy, might be more realistic. This state of "post-treatment control" has been observed in a small proportion of patients and has become an important focus of HIV cure research. The main goal of this doctoral thesis was to find immunologic and/or viral biomarkers which are predictive of functional cure with the help of three clinical trials.

In the first trial we hypothesized that virally suppressed patients on cART who have a very small viral reservoir (measured as the amount of proviruses) and very little proviral transcriptional activity (measured as the amount of cell-associated viral RNA), would be more likely to have delayed viral rebound after treatment interruption as compared to the general HIV positive population on cART. Sixteen such patients interrupted therapy but all experienced rapid viral rebound within two to eight weeks. This observation is consistent with the findings from other recent trials and affirms that it is unlikely that post-treatment control can be

achieved by merely reducing the size of the reservoir and that an immune intervention is required.

In the second trial we set up a sensitive and specific assay to measure the *in vitro* viral inhibitory activity (VIA) of CD8+ T cells, which was to be used in the third (therapeutic vaccination) trial. In addition, we explored associations between VIA and clinical, viral and immune parameters. Interestingly, we found positive associations of VIA with expression of immune exhaustion markers PD1 and CD160, as well as with CD57, a marker of replicative senescence, and HLA-DR, a marker of immune activation, on terminally differentiated memory CD8+ T cells. While we did not show that these cells are responsible for the VIA we measured, it indicates that a distinct activation state of fully differentiated memory cells might play an important role in suppressing viral replication.

In the third and last trial, we assessed the immunogenicity of an innovative therapeutic vaccination strategy and its capacity of inducing delayed viral rebound after treatment interruption in virally suppressed patients on cART. Study participants received three intra-nodal injections of a naked mRNA vaccine. The mRNA construct coded for a set of rationally selected, conserved, subdominant HIV peptides (vaccine) and three dendritic cell activation stimuli (adjuvant). Unfortunately, the construct was found to contain an error which likely compromised the translation of the mRNA, thereby invalidating the findings of the trial.

In conclusion, the findings from this doctoral work contribute to the growing consensus that the induction of a functional cure will require an immune intervention (e.g. therapeutic vaccination) on top of a sufficiently reduced viral reservoir. Unfortunately, we were not able to verify the potential of our own therapeutic vaccine due to a serious error in the mRNA construct which was produced by one of our private partners.

Samenvatting

Terwijl combinatie antiretrovirale therapie (cART) er zeer goed in slaagt om virale replicatie te onderdrukken en het natuurlijke ziekteverloop af te stoppen, verhindert de aanwezigheid van een latent viraal reservoir in rustende geheugen CD4+ T cellen de volledige genezing van een HIV infectie. HIV positieve patiënten dienen bijgevolg levenslang medicatie te nemen. Behalve de hoge kostprijs van deze medicatie, blijkt het ook bijzonder moeilijk om alle HIV patiënten op behandeling te krijgen, vooral in landen met een laag en gemiddeld inkomen. Deze dagelijkse medicatie beïnvloedt daarenboven de levenskwaliteit van de patiënt, ook al is de behandeling vandaag gereduceerd tot een pil per dag, en zou desalniettemin op lange termijn een impact kunnen hebben op de gezondheid van de patiënt. Een echte genezing voor HIV is daarom wenselijk.

Het wordt alsmaar duidelijker dat het bereiken van een 'steriliserende genezing', waarbij een patiënt volledig virus-vrij wordt, zeer moeilijk wordt. Een 'functionele genezing' daarentegen, waarbij een patiënt nog steeds geïnfecteerd is maar het virus onderdrukt zonder de behoefte aan cART na een periode van behandeling, lijkt een meer haalbaar alternatief. Dit fenomeen van 'post-behandeling controle' is reeds waargenomen in een kleine proportie van patiënten en is ondertussen een belangrijke focus binnen het HIV cure onderzoek. Het centrale objectief van deze thesis was het zoeken naar immunologische en/of virale biomerkers die predictief zijn voor een functionele genezing van HIV, aan de hand van drie klinische trials.

De hypothese van de eerste trial luidde dat de kans op een vertraagde virale rebound bij viraal onderdrukte patiënten die op behandeling staan, een klein viraal reservoir hebben (gemeten als het aantal provirussen) en een zeer beperkte provirale transcriptionele activiteit vertonen (gemeten als de hoeveelheid cel geassocieerd viraal RNA) kleiner zou zijn dan bij de algemene

patiëntenpopulatie op behandeling. Zestien zulke patiënten onderbraken hun behandeling maar vertoonden allen een snelle virale rebound binnen twee tot acht weken. Deze bevinding is consistent met resultaten uit andere recente trials en bevestigt dat een functionele genezing waarschijnlijk een immuun interventie zal vereisen en niet kan bereikt worden enkel en alleen door het viraal reservoir te verkleinen.

In de tweede trial werd een sensitieve en specifieke assay opgezet die de *in vitro* virale inhibitorische activiteit (VIA) meet van CD8+T cellen, dewelke in de derde (therapeutische vaccinatie) trial gebruikt zou worden. Daarenboven werden associaties onderzocht tussen VIA en klinische, virale en immunologische parameters. Een opmerkelijke correlatie werd gevonden tussen VIA en de expressie van immuun uitputtingsmerkers PD1 en CD160, evenals CD57, een merker van replicatieve ouderdom, en HLA-DR, een immuunactivatiemerker, op terminaal gedifferentieerde geheugen CD8+ T cellen. Hoewel er niet werd aangetoond dat deze cellen effectief verantwoordelijk zijn voor de gemeten VIA, wijst dit wel op een belangrijke rol van volledig gedifferentieerde geheugencellen met een welbepaalde staat van activatie bij het onderdrukken van virale replicatie.

In de derde en laatste trial, werd de immunogeniciteit van een innovatieve therapeutisch vaccinatiestrategie geëvalueerd, evenals diens capaciteit om een virale rebound te vertragen na het onderbreken van de behandeling bij viraal onderdrukte patiënten op cART. Studieparticipanten kregen drie intra-nodale injecties van een naakt mRNA vaccin. Dit mRNA construct codeerde voor een aantal rationeel geselecteerde, geconserveerde, subdominante HIV peptiden (vaccin) en drie activatiestimuli van dendritische cellen (adjuvant). Er werd echter een belangrijke fout gevonden in het construct waardoor de translatie van het mRNA

waarschijnlijk niet efficiënt is verlopen, hetgeen de resultaten van de trial onbruikbaar hebben gemaakt.

Alles bij elkaar genomen hebben de resultaten van dit doctoraal onderzoek bijgedragen tot de consensus dat het induceren van een functionele genezing van HIV, naast een verkleining van het viraal reservoir, een immuun interventie vereist (zoals een therapeutische vaccinatie). Helaas hebben we het potentieel van ons eigen therapeutisch vaccin niet kunnen evalueren door de aanwezigheid van een belangrijke fout in het mRNA construct dat geproduceerd werd door een van onze private partners.