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Development and evaluation of a heterogenous virus-like particle (VLP) formulation to achieve HIV-1 latency reversal and cure.

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Abstract

HIV-1 is the etiological agent behind acquired immune deficiency syndrome (AIDS) – a chronic, life-threatening condition that compromises host immune function. After nearly four decades and despite ongoing global efforts, HIV-1 persists in nearly 38 million individuals worldwide. Of this population, only 60% have access to life-saving combination antiretroviral therapy (cART), clearly emphasizing the need to realize a cure. Unfortunately, the establishment of replication-competent provirus in resting CD4+ T lymphocytes represents a significant barrier to HIV-1 curative research. The viral reservoir is highly stable and has a half-life of ~44 months. Therefore, it is unlikely that infection will naturally exhaust over the course of a human lifetime. Furthermore, infected cells are phenotypically indistinguishable from uninfected CD4+ T cells – thus making it difficult to selectively target these cells for eradication. Evidence suggests that HIV preferentially infects and establishes latency within HIV-specific CD4+ T cells and that HIV latency reversal can be achieved using HIV derived proteins. Therefore, a therapeutic vaccine that represents the entire proteome of HIV within a given individual, immediately prior to antiretroviral therapy, might activate the entire cellular reservoir and initiate proviral gene transcription. Herein, we investigate the ability of a highly diverse virus-like particle formulation to 'shock' HIV-infected cells into transcriptional activity, thus leading to their eradication via immune-mediated or viral cytopathic effects. This activation vector (ACT-VEC) represents the first targeted approach to HIV-1 latency reversal. Based on the detection of viral RNA in culture supernatants, we show that ACT-VEC significantly outperforms other clinically relevant latency reversing agents at both the acute and chronic stages of infection. Using a quantitative outgrowth assay, we determined that ACT-VEC was also capable of inducing replication competent provirus from HIV-infected CD4+ T cells. Furthermore, we provide preliminary evidence that a virus-like particle formulation can provide an immune-mediated 'kill' after transcriptional reactivation occurs. Our VLP construct is also minimally antigenic, suggesting that it will be well tolerated *in vivo.* All together, our research suggests that ACT-VEC is a highly efficacious transcriptional reactivator that merits further investigation in the context of curative therapeutic strategies.

Summary for Lay Audience

Human immunodeficiency virus 1 (HIV-1) is a virus that, in the absence of treatment, leads to acquired immunodeficiency syndrome (AIDS). AIDS is typically described as a period during which a subclass of immune cells, called T cells, die. In the absence of T cells, an HIV-infected individual is unable to mount a proper immune response and can be at an increased risk of infection by other pathogens (ie. bacteria, viruses, etc.). These infections, which are often uncommon in humans with properly functioning immune systems, lead to AIDS-related death. Despite efforts to stop HIV-1, the virus continues to persist in approximately 38 million individuals worldwide. Of this, approximately 60% have access to lifesaving medications which function to inhibit the virus' life cycle. Unfortunately, several factors ultimately prevent these medications from being a long-term feasible solution, including i) high therapy-associated cost, ii) the requirement to take medication daily, for life, iii) logistical barriers, and iv) economic barriers. Furthermore, the currently available medications are non-curative due to the ability for HIV-1 to persist within host immune cells. There remains a need for new strategies capable of removing cells harboring HIV-1. The literature suggests that the reservoir can be targeted using small, HIV-1 proteins. These proteins are presented to cells that have a high likelihood of harboring latent HIV-1 and, subsequently, can stimulate the target cell. After reactivation, cells can be targeted by the immune system for destruction. Herein, we present an activator vector (ACT-VEC) that contains proteins encompassing the entirety of HIV-1. This activator vector currently outperforms all clinically-relevant drugs designed to remove latent HIV-1. ACT-VEC causes minimal immune activation, which suggests that it will be well tolerated in further stages of testing. Overall, we present a novel vaccine formulation capable of removing latent virus from host CD4 T cells. The activator vector represents a novel strategy for achieving HIV-1 cure and merits further investigation.

Co-Authorship Statement

The results presented herein are predominately carried out by Joshua Pankrac, in collaboration with the laboratories of Dr Jamie F.S. Mann and Dr Eric Arts. Additional information regarding collaboration is as follows:

Chapter 2: <u>Pankrac, J.</u>, Klein, K., McKay, P.F. et al. A heterogeneous human immunodeficiency virus-like particle (VLP) formulation produced by a novel vector system. npj Vaccines 3, 2 (2018). https://doi.org/10.1038/s41541-017-0040-6

JFSM, JP, RS, DC, SF, and EJA designed experiments. JFSM, JP, YG, KK, and TB built the VP, VLP and ACT-VEC constructs. JP, KK, JFSM performed all qRT-PCR experiments and analysis. JP, KK, and KB performed deep gene sequencing and both JFSM and KB performed all the radioactive RT assays. KK and EJA performed NGS analysis. JK and JP did Veritrop assays. JP did the zeta sizing and electron microscopy work. SF, RS, PM and DK, recruited, consented and processed blood samples. JFSM, KK and JP did the immunogenicity studies and JP, KK, JFSM and EJA wrote the manuscript. The manuscript was reviewed by the authors.

Chapter 3: Jamie F.S. Mann, <u>Joshua Pankrac</u>, Katja Klein, Paul F. McKay, Deborah F.L. King, Richard Gibson, Chanuka N. Wijewardhana, Rahul Pawa, Jodi Meyerowitz, Yong Gao, David H. Canaday, Mariano Avino, Art F.Y. Poon, Caroline Foster, Sarah Fidler, Robin J. Shattock and Eric J. Arts (2020). A targeted reactivation of latent HIV-1 using an activator vector. *Manuscript Accepted (EBioMedicine).*

JFSM, JP, RG, DHC and EJA designed experiments. JP, RP, and CNW performed quality control for VP and VLP formulations. JP and RP performed all qRT-PCR experiments and analysis. KK and JP prepared samples for Illumina sequencing. MA, AFYP, and JFSM analyzed Illumina data. JP performed the immunogenicity assays. JFSM and JP performed the viral outgrowth-like assay. SF, RS, PM and DK, recruited, consented and processed blood samples from HIV-infected donors. JP, CNW, and JFSM recruited, consented and processed blood from HIV-seronegative donors. JFSM, JP, KK, and EJA wrote the manuscript. All authors reviewed the manuscript.

Chapter 4: <u>Joshua Pankrac</u>**, Jamie F.S. Mann, Emmanuel Ndashimye, Rahul Pawa, Renata Ceccacci, Eric Arts.**

The work presented herein reflects ongoing work within the lab. JP, JFSM, EA designed the experiments. JP made Sub-B VLPs. EN and RP made Sub-D VLPs. JP performed the latency reversals and ELISpot assays. JP and RC performed qRT-PCRs. JP wrote the chapter. JP, JFSM edited the chapter.

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Table of Contents

Table of Figures

Chapter 1 - Introduction

1.1 A Landscape of HIV-1 Infection

1.1.1 A Timeline of HIV-1 Discovery

On June 5, 1981, a report was released by the United Stated Centers for Disease Control and Prevention (CDC) describing an unusual case of *Pneumocystis carinii* in five previously healthy men, all of whom had sex with men (MSM), in the Los Angeles area.[1] The occurrence of these cases was particularly notable as *Pneumocystic* pneumonia is usually only prevalent in highly immunocompromised individuals, leading medical experts to wonder what etiological agent had led to the disease's onset.[2] In the ensuing days and weeks, numerous reports were filed with similar cases, all of which occurred within the MSM community. This finding, in conjunction with the occurrence of Kaposi's Sarcoma, an aggressive cancer caused by various Herpesviruses, in 41 homosexual men, led to many individuals referring to the condition as 'Gay-Related immune deficiency' or GRID. By the end of the year, 121 individuals had succumbed to the illness. The cause of these deaths remained elusive and engrained a public stigma towards the MSM community – already, what is now known as HIV-1, was causing panic and devastation.

It was not until 1983 that the CDC began reporting cases in female sexual partners of individuals with Acquired Immune Deficiency Syndrome (AIDS).[3] To add to this observation, a subsequent report further noted cases in intravenous drug users, Haitians, and hemophiliacs.[4] This revelation changed the AIDS paradigm. Suddenly, a disease of the homosexual community had become one more likely associated with a sexual or blood-associated pathogen. That year, Dr. Luc Montagnier and Dr. Barré-Sinoussi, researchers at the French Pasteur Institute, along with colleagues, were the first to identify a T cell tropic retrovirus within a lymph node biopsy of an individual preceding AIDS.[5] At the time, this retrovirus was called Lymphadenopathy Associated Virus (LAV). In April of 1984, Dr. Robert Gallo confirmed that a retrovirus, which he termed HTLV-III, was indeed the etiological agent responsible for AIDS and was identical to the virus isolated in France.[6] The ambiguous nature of the viruses name, HTLV-III/LAV, was addressed in 1986, when the International Committee on the Taxonomy of Viruses agreed upon a unifying name, Human

Immunodeficiency Virus (HIV).[7] Montagnier and Barré-Sinoussi, went on to receive the Nobel Prize for the discovery of HIV-1 in 2008.[8]

Despite the 1980s being a seminal period regarding HIV identification and characterization, the first transmission event likely occurred several decades prior. In fact, HIV-1 viral sequences were detected as early as 1959 in plasma samples collected from an African individual.[9] The finding suggests that an early transmission event, one that pre-dated the Los Angeles cases, likely occurred in the geographical area. A supporting study of the V3-V5 Env region of HIV implicated Central Africa and, specifically, the Democratic Republic of Congo, as a 'hotspot' for HIV-1 group M genetic diversity.[10] The high level of cocirculating subtypes and recombinant viral forms implicate this region as a pivotal area for HIV-1 dispersal. Interestingly, researchers observed that HIV shares many commonalities with Simian Immunodeficiency Virus (SIV), and both preferentially target and infect lymphocytes in a mechanistically similar way. With evidence of zoonotic transmission between various non-human primates (NHPs), it was realistic to conclude that such a transmission event could occur between NHPs and humans, as well. The 'hunter' theory hypothesized that transmission occurred when a human hunter became exposed to the SIV-infected blood of his/her NHP prey.[11] Although there have likely been multiple cross-species transmission events throughout time, most events likely failed to disperse throughout the general population due to host defense mechanisms. However, a single well-adapted viral transmission event from chimpanzees (SIV_{cpz}) is thought to be responsible for the zoonotic transmission of the AIDS-associated, HIV-1 group M viruses.[12–14]

1.1.2 The Global Burden of HIV-1 – From Past to Present

Since the beginning of the epidemic, HIV-1 has infected approximately 75 million individuals worldwide.[15] Today, an estimated 37.9 million individuals live with HIV-1 infection, wherein 1/5th, or 8 million people, are unaware of their HIV-1 status despite increasing global efforts. Notwithstanding, the advent of combination anti-retroviral therapy (cART) has transformed the HIV-1 death sentence into a chronic lifelong illness for approximately 23 million people with access to these life-saving drugs. As a greater proportion of individuals begin accessing cART, year-to-year mortality from AIDS-related death is also declining. From the period of 2016 to 2019, mortality has declined by approximately 25%, from 1.1 million reported deaths to 770 000

deaths, respectively.[15,16] Though the impact of these multi-national efforts are noticeable, there is still much that needs to be done, and UNAIDS still strives to achieve their '90-90-90' goal, despite failing to do so by their original 2020 deadline. This goal pushes for 90% of HIV-infected individuals to i) know their HIV status, ii) have access to cART and, iii) retain prolonged viral suppression. Such efforts come at a significant cost, however, with US$ 19.0 billion in AIDS relief in 2018. Clearly, HIV-1 has established itself amongst the most devastating infectious diseases to date, and great effort has been invested into the identification of novel strategies for prevention and cure. Such efforts hope to address the HIV-1 epidemic in both a more sustainable and accessible manner.

1.2 Characterization of the HIV-1 Virus
1.2.1 The Structural and Genomic Makeup of HIV-1
Human Immunodeficiency Virus (HIV) is taxonomically classified as a member of the genus *Lentivirus* and the family, *Retroviridae*. The HIV genome is comprised of two positive-sense, single-stranded RNAs (ssRNAs) within a conical capsid composed of virally-derived p24 protein.[17] The 9.7 kb genome encodes for sixteen (16) viral proteins, which can be stratified into structural, regulatory, or accessory proteins.[18] As with all members of the Retrovirus family, HIV contains three polyproteins, Gag, Pol, and Env, which serve prototypic functions regarding structure, enzyme synthesis, and envelope formation, respectively.[18,19] Specifically, the Gag gene encodes for structural matrix (p17), capsid (p24), nucleocapsid (p7), p6, and spacer proteins which comprise all necessary components for virus-like particle formation and nuclear genomic stabilization.[20–22] In contrast, the Pol region encodes for viral enzymes, such as reverse transcriptase, ribonuclease H, protease, and endonuclease, which are implicated in viral maturation and infectivity.[23] The final polyprotein, Env, is responsible for viral entry via the production of envelope glycoprotein, gp120, and its associated protein, gp41. These proteins ultimately determine viral tropism and mediate fusion between the virus and target cell.[24] To incorporate the necessary proteins involved in viral infection and production of progeny virus, HIV employs alternative splicing, a process that produces more than 100 different mRNAs from a single primary RNA transcript.[25,26] For alternative mRNA production to occur, HIV utilizes the cellular spliceosome, which can recognize several unique 5' and 3' splicing motifs to produce

alternative resultant mRNAs.[27,28] Using this method, splicing can produce both singly-spliced (~2kb) and multiply-spliced (~4kb) mRNAs.[29] The production of the regulatory proteins, *tat* and *rev*, as well as the accessory protein, *nef*, are produced via complete splicing initiated at 3' motifs located centrally within the RNA genome, followed by subsequent translation in the cell cytoplasm.[25,29] Briefly, Tat protein functions through binding of the trans-activation response (TAR) element and recruitment of the RNA Polymerase II elongation factor, P-TEFb, which is necessary for viral mRNA production.[30,31] The regulatory protein, Rev, engages with a cis-acting Rev response element (RRE) to facilitate RNA trafficking and nuclear export to the cell cytoplasm.[32–34]

Accessory proteins, including *vif, vpr, vpu,* and *nef*, have been well-characterized in regards to their involvement in immune evasion, antiviral resistance, replication, and dissemination.[35] These proteins often modify the local environment to increase suitability for the infecting virus. The *nef* protein is expressed early after onset of infection and is heavily implicated in pathogenicity, as long-term survival is seen in individuals infected with HIV-1Δ*nef* mutants in the absence of cART.[36] Furthermore, *nef* downregulates cell surface proteins, such as CD4, through an interaction with the protein's cytoplasmic tail, and subsequently transports the protein to the endosome for degradation.[37,38] The function of downregulating host CD4 is also seen in *Vpu*, although the mechanism and location of the interaction differs from *nef*.[35] Furthermore, *Vpu*, and *Vif* are capable of suppressing host restriction factors such as the lipid raft protein, tetherin (BST-2), and the cytidine deaminase, APOBEC3G, respectively.[39,40] Similarly, *Vpr*, despite some ambiguity on various functions, is surely implicated in G2 cell-cycle arrest via the engagement of a cullin-RING ubiquitin ligase, DCAF1.[41,42] The summative function of these accessory proteins allows for continued persistence of HIV-1 through interruption of host immune mechanisms otherwise designed to clear infection.

1.2.2 HIV Infectious Cycle
It is important to acknowledge the contribution of the HIV-1 viral proteins in the context of the virus' replication cycle during natural infection (**Figure 1-1**). By 'natural', this refers to HIV-1 infection in the absence of cART. The replication cycle of HIV-1 can be stratified into early and late phases. The early phase is defined as the period from viral entry up until proviral integration

in the host genome. Subsequently, late phase refers to the period of proviral transcription up until release of HIV-1 progeny. The duration of this entire process takes approximately 1-2 days and, during this time, is capable of producing up to $2x10^9$ progeny virions per day.[43] The resulting virus lacks uniformity due to the extremely high mutation rate (1/10 000 nucleotides) of the virally-encoded reverse transcriptase enzyme. Because of this, HIV-1 infection is not typically characterized by a single viral clone, but rather as quasi-species comprised of many HIV variants. The high sequence variability within the HIV quasi-species contributes to difficulties achieving efficacious vaccine strategies, as each viral variant can potentially exhibit altered peptide antigens. Thus, the pool of target cells becomes exponentially higher than in the case of a single viral clone. It is worth noting, however, that many mutations are lethal and prevent infectious particle formation and release. In support of this, a 44-fold decrease in mutation rate was observed in viral sequences derived from supernatants as compared to those derived intracellularly, suggesting a high occurrence of deleterious mutations.[44]

1.2.3 The Early Phase of Replication

Due to the short half-life of cell-free infectious virions – approximately 20-30 minutes – it is imperative that HIV-1 can rapidly identify and infect permissive cellular targets.[43] Viral attachment is determined through targeting of CD4, the primary receptor, and one of two co-receptors, CCR5 or CXCR4. Cells expressing the appropriate receptor/co-receptor are susceptible to infection, although attachment is typically inefficient due to low Env density on the HIV-1 surface. Following attachment, fusion is facilitated through an interaction with virally-derived gp120 and host CD4. This binding interaction creates a conformation change within the Env protein trimer, which subsequently binds to either the CCR5 or CXCR4 co-receptor. Following co-receptor engagement, another conformational change occurs which reveals the gp41 transmembrane protein. The fusion peptides present on gp41 can interact with the host cell and ultimately allows gp41 to create a 6-helical bundle which facilitates viral-cell fusion. Upon membrane fusion, viral contents can freely be released into the cell. Virally-derived positive-sense RNA, with the help of the reverse transcriptase enzyme, can then utilize cell-host machinery to reverse transcribe into dsDNA. HIV uncoating likely occurs during the transition from reverse transcription to loading into pre-integration complexes (PICs).[45] The PIC is
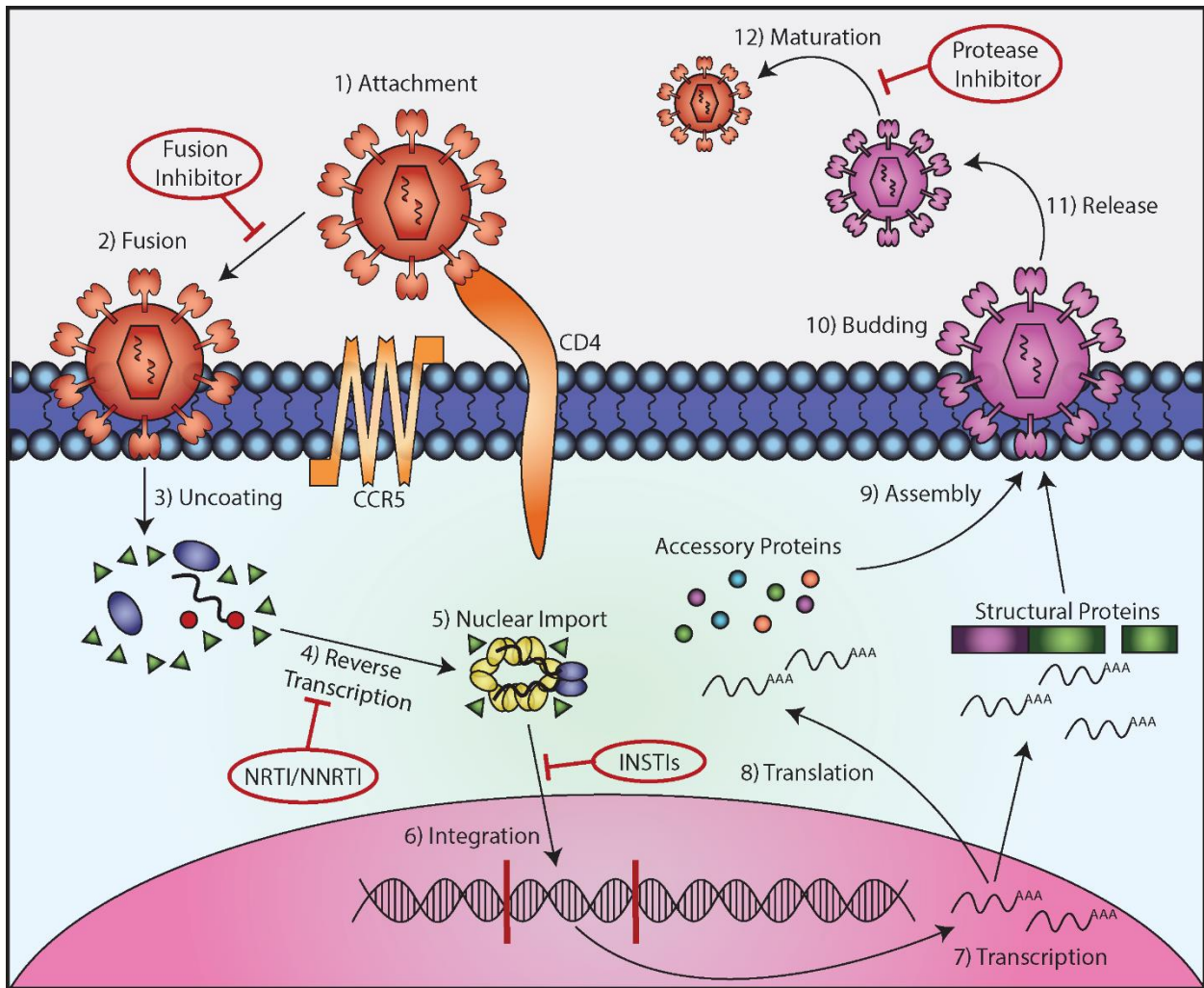
Figure 1-1: A schematic of the HIV-1 life cycle and points of therapeutic inhibition

The HIV-1 life cycle is a multi-step process which can be divided into distinct phases, as denoted by the numbering (1-12) above. The early and late phases of viral replication are denoted by numbers 1-6 and 7-12, respectively. The classes of antiretroviral drugs designed to target specific stages of the life cycle are denoted by red circles. NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; INSTI, integrase strand transfer inhibitor.

transported across the nuclear membrane via a nuclear pore. Through utilization of the protein, integrase, viral dsDNA can incorporate itself into the host cell genome.

Integration into the host genome is a critical time for HIV-1 and, specifically, strategies targeting the virus for eradication. It is at this point that the virus has two fates, depending on the status of the infected host. The first fate is to continue into the late phase of viral replication, in which new viral RNA is transcribed along with the host genome. The alternative, however, is that integration occurs in the absence of late phase replication due to low transcriptional levels within the host. It is in this second scenario, where HIV-1 has successfully integrated but remains transcriptionally inactive, that viral latency occurs. Viral latency continues to persist for the lifetime of the infected cell.

1.2.4 The Late Phase of Viral Replication

During productive infection, HIV provirus utilizes cellular Pol II polymerase to transcribe messenger/genomic RNA. Transcriptional initiation and elongation begin at the viral promoter site present within the HIV-1 5'LTR U3 region but, initially, viral transcription is limited due to inefficient elongation. Therefore, transcriptional elongation is largely dependent on viral Tat protein, which provides a positive feedback loop to facilitate efficient processing. Tat-mediated transcriptional synthesis ultimately produces full-length transcripts and several different mRNAs with various degrees of splicing. mRNA products are then transported to the cell cytoplasm via the viral Rev protein. Within the cytoplasm, full-length mRNAs expressing Gag and Gag-Pol precursors are then processed into key structural proteins. Following multimerization of Gag and Gag-Pol-derived proteins, viral Env glycoproteins and genomic RNA are recruited to the assembling virion. Immature virions subsequently bud from the surface of the cell and, only after fully budding, mature via processing of the Gag and Gag-Pol precursors.

1.2.5 HIV Entry and Infection

Sexual intercourse is by-and-large the main source of HIV-1 spread worldwide and has been implicated in 70-80% of total infections worldwide. Despite this, the rate of infection per coital act is remarkably low, with likelihoods of 0.06% and 0.01-1% for anal and vaginal penetration, respectively. Surely, an interplay of variables must explain the discrepancy between the high contribution to total infections and the low risk of exposure. By looking at the site of primary

infection, it is possible to determine many of the factors that may contribute to HIV-1 susceptibility.

Initially, HIV-1 must penetrate the vaginal epithelium and access underlying target cells that are permissive to infection. The vaginal epithelium varies from thick, stratified squamous epithelium in the ectocervix to single layered columnar cells within the vaginal endocervix. The thickness of the vaginal ectocervix poses a difficult physical barrier for HIV-1 to overcome, and contains a subset of DCs called Langerhans cells. However, unlike traditional DCs that are implicated in viral presentation to CD4 T cells, Langerhans cells inhibit HIV-1 infection via the type II transmembrane protein and C-type lectin receptor, Langerin.[46] When T cells were exposed to low concentrations of virus in the presence of various DC subsets, only Langerhans cells prevented efficient infection from occurring.[46] At higher concentrations, infection could efficiently establish itself without DCs, but was still inhibited by the presence of Langerhans cells which internalized and degraded HIV-1 in Birbeck granules. Due to overwhelming immune pressure present within the ectocervix, the current belief remains that HIV accesses target cells through micro-abrasions in the columnar epithelium of the endocervix. The virus can then disseminate via locally draining lymph nodes, thus establishing systemic infection.

1.3 Understanding HIV-1 Latency
1.3.1 Establishment of the Latent Reservoir
A difficulty with defining the mechanism behind HIV-1 latency is the inability to study establishment *ex vivo*, as, by definition, cells isolated from an infected individual will already be latent.[47] Despite this, studies of resting cells harboring integrated provirus provide us with an invaluable tool on how this process might occur. Resting memory CD4 T cells harboring integrated provirus are of notable importance, due to their prolonged lifespan relative to other cellular subsets.

When a dendritic cell interacts with a T cell via antigen presentation, naïve CD4 T cells become activated and subsequently undergo differentiation into various effector T cell subsets: Th1, Th2, Th17, and T_{reg} cells.[48] At this point, effector cells responding to infection represent highly susceptible targets for viral entry and proviral integration. Most of these cells will subsequently undergo productive infection, during which the production of viral transcripts and proteins

occurs **(Figure 1-2)**. This productive cellular subset is primed for clearance via immune-mediated or cytotoxic effects and, therefore, is unlikely to contribute to HIV persistence *in vivo*.[49,50] Rather, viral latency most likely occurs within 'resting' cells with low metabolic states. Of importance are resting memory CD4 T cells, which are distinguishable from activated cells through i) decreased expression of activation markers, ii) low RNA content, iii) smaller cell size and, iv) a lack of cell cycling.[51] The combination of low transcriptional activity and high lifespan of these memory T cells provides an optimal environment for prolonged viral persistence. Clearance of these cells through immune-mediated mechanisms is difficult to achieve due to the absence of viral proteins and the prevalence of escape mutations within the integrated provirus.[52]

There are two distinct methods which may contribute to the establishment of latency within resting memory CD4 T cells. As alluded to previously, the first method is through viral infection of effector T cells which subsequently revert to a resting memory state. This is commonly accepted as the most predominant method of latency establishment, as effector cells exhibit greater permissiveness than resting cells. If this theory is true, most HIV provirus should persist within the memory CD4 T cell reservoir, making these cells a priority target for novel vaccine regimens.[47] Alternatively, latency could also result from the direct infection of resting cells, which could include either naïve or memory subsets. In this model, the viral reservoir would persist in an expanded, less specific pool of cells. Although this is a potential explanation, factors including low CCR5 expression, limited dNTP availability, and poor integration efficiency all contribute to worsened efficiency of infection.[53–55]

The memory CD4 T cell reservoir represents the *predominant* HIV-1 reservoir, although it is worth mentioning that other cellular subsets have been shown to harbour latent provirus, as well. Two other T cell subsets, naïve CD4 T cells and γδ T cells, have also been shown to harbour latent provirus.[56,57] Furthermore, *in vitro* studies have identified macrophages as permissive target cells, however, it is uncertain whether macrophages can harbour HIV provirus in the presence of cART.[58] The contribution of each of these subsets of cells towards the latent reservoir is controversial, as they either have a short half-life or do not produce daughter cells to carry on infection.
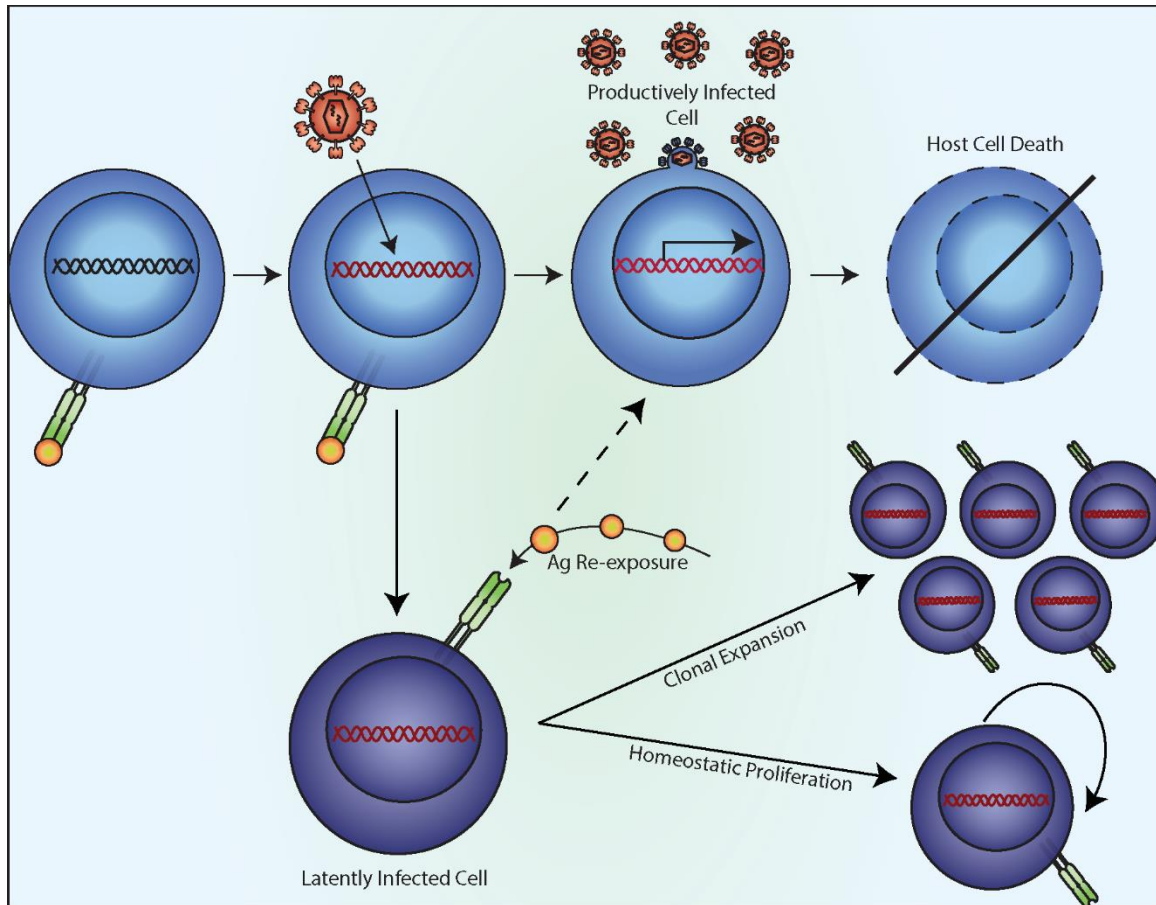
Figure 1-2: Establishment of quiescent HIV-1 within host CD4 T cells.

Permissive host CD4 T cells are susceptible to HIV-1 infection and integration. These cells have two fates, productive infection (top) or latent infection (bottom). Transcriptionally active, infected cells produce viral proteins and progeny virions and subsequently lead to host cell death. Latent cells (purple) are devoid of transcriptional products and can reactivate in the presence of antigen re-exposure. Latent cells are maintained through clonal expansion and homeostatic proliferation.

1.3.2 The Contribution of HIV-1 Tat to Latency

Out of all the proteins produced by HIV-1, the Tat protein is the most heavily implicated in the establishment and maintenance of viral latency. Specifically, through the regulation of RNAP II elongation on the viral 5' LTR, Tat functions as the virus' transcriptional switch. To initiate viral transcription, Tat must bind the TAR hairpin present within the RNA transcript, while simultaneously engaging with p-TEFb, composed of Cyclin T1 (CycT1) and cyclin-dependent kinase 9 (CDK9). This interaction leads to the phosphorylation of C-terminal RNAP II, thus initiating transcriptional elongation at the promoter site.[59] In studies evaluating HIV infection in the absence of functional Tat, transcripts were short (<100 nucleotides), non-polyadenylated, and contributed to a decrease in viral replication capacity.[59,60] Such transcriptional deficiencies reveal that the Tat protein serves as a virally-endogenous mechanism to mediate the productive or quiescent status of HIV-1.

The Tat protein poses a challenge to a commonly accepted hypothesis that latent infection is controlled exclusively through environmental factors, such as cellular metabolic state. In this model, HIV latency is merely a tangential phenomenon arising from a cellular transition between active and resting states. In actuality, this does not appear to be the case. In a study by Razooky et al., the Tat-positive feedback circuitry was sufficient to induce viral transcription in the absence of cellular activation.[61] This means that a virally-encoded Tat-dependent mechanism, rather than the cellular environment, sufficiently regulates viral transcription and, in essence, latency. More specifically, positive feedback of Tat is critical for productive infection to occur. To show this, a study by Lassen et al. revealed that a break in the Tat-mediated feedback loop via the retention of HIV msRNA encoding tat/rev contributed to non-productive infection *ex vivo*.[62] Alternatively, the removal of host factors responsible for the interruption of Tat's translocation activity, such as non-coding RNAs, lead to a pronounced increase in proviral reactivation within HIV-infected CD4 T cells.[63] Finally, the introduction of exogenous Tat into the Jurkat cell line, a model for HIV latency, was also shown to recover transcriptional reactivation within the latent population.[64] It is difficult to dismiss the evident role Tat plays in HIV-1 latency and, by extension, transcriptional reactivation of dormant provirus. For this reason, emphasizing strategies which maintain or assist

in Tat-dependent feedback are of considerable value when developing latency reversing regimens.

1.4 The Search for a Cure

1.4.1 CCR5Δ32 Hematopoietic Stem Cell Transplantation (HSCT)

To date, there are two reported events of HIV-1 cure, the Berlin Patient and the London Patient.[65–67] These seminal cases provide hope that an HIV-1 cure will be realized, however, they provide limited feasibility in most other cases due to i) procedure-associated risks, ii) high procedure-associated costs and, iii) limited scalability. Take the case of Timothy Ray Brown, more commonly known as the Berlin Patient. Mr. Brown had chronic HIV infection when he was diagnosed with acute myeloid leukemia. During the treatment, Timothy received a hematopoietic stem cell transplant from a patient with homozygous, CCR5Δ32 alleles – a deletion that ultimately renders CCR5-tropic viruses, such as most HIV-1 variants, unable to infect target cells.[65] Importantly, the procedure's success relied on a stem cell transplant preceded by irradiation of immune cells to prevent graft-versus-host disease. In other words, Timothy lacked a functional immune system. Typical pathogens, ones that commonly go unnoticed due to rapid clearance by immune-mediated mechanisms, could freely infect Timothy during this time. This, along with the procedure itself, nearly killed Mr. Brown. Clearly, for most individuals, the procedure itself is inhibitory in nature, especially with the prevalence of life saving combination antiretroviral therapy (cART) that can be taken indefinitely with minimal risk. The success of the Berlin Patient was consequently translated into a second success, the London Patient. Like its former, this individual was HIV-positive while being treated for Hodgkin's Lymphoma, and similarly underwent a homozygous CCR5Δ32 stem cell transplant. Importantly, the London Patient received a less toxic regimen, i.e. lymphoma-targeting chemotherapy agents, alone. Although the London Patient experienced grade-1 graft-versus host disease, followed by viral recrudescence of Epstein-Barr virus (EBV) and cytomegalovirus (CMV) several months post-transplant, the effects were less severe than that of the Berlin counterpart.[67] As of April 2019, the London Patient has been in remission for 18 months (<1 HIV copy/mL).

Other attempts have been made to replicate the success of the Berlin and London Patients, however, several factors have posed limitations to the aforementioned approach. Although the

procedure has been repeated at least half a dozen times since the Berlin Patient, four out of six (4/6) patients died shortly thereafter due to either i) transplant-associated infections or, ii) cancer relapse.[68] The other two instances provide learning moments regarding the complicated nature of transplant-associated cure strategies. Of note, the presence of a secondary co-receptor for HIV-1, CXCR4, provides an alternative avenue for target cell entry in the absence of CCR5, and may provide a viral escape mechanism that undermines the CCR5 knockout strategy.[69,70] One such instance occurred in the Essen Patient, a CCR5 knockout transplant recipient being treated for anaplastic large-cell lymphoma.[70] Viral RNA extracted from this individual prior to transplant indicated that the infecting virus was largely CCR5-tropic, based on analysis of the HIV-1 Env V3 region. However, proviral DNA extracts from the same donor had an intermediate phenotype – a mixture of CCR5 and CXCR4-tropic viruses. After 3 weeks post-transplant, the Essen patient had to re-initiate ART due to a substantial rebound in viremia (93,390 copies/mL).[70] This viral escape mechanism, wherein the infecting virus utilizes an alternative co-receptor, complicates transplant-based cure strategies. Indeed, the viability of a co-receptor-centric approach may require a dual-knockout of both CCR5 and CXCR4 to confer absolute resistance to HIV-1 infection.[71,72] The failure of the Essen patient is further confounded by variability in the transplant methodology. Hütter discerns between the Berlin and Essen patients on one particular metric, the presence or absence of prolonged ART during transplantation.[68] Perhaps, in the Berlin case, the extended duration of ART contributed to stabilization of donor transplant cells – something that was absent in the Essen case. Furthermore, in addition to finding homozygous CCR5Δ32 donors, HLA-matching between donor and recipient will surely contribute to graft success.[73]

Although evidence strongly suggested that the homozygous CCR5Δ32 deletion was a fundamental characteristic of a successful HSC transplant, a subsequent study was performed in two individuals receiving transplants with wild-type co-receptor alleles.[74,75] Of interest, Henrich et al. sought to determine if a wild-type HSCT could contribute to viral reservoir reduction in the prolonged absence of cART. If true, this finding would offer a greatly expanded pool of potential donors, as finding individuals with homozygous knockout mutants remains a bottleneck in the treatment pipeline. Unsurprisingly, however, rebound viremia occurred within 12 to 32 weeks,

respectively, upon cART cessation.[75] Therefore, inhibition of viral entry mechanics via co-receptor modulation is surely implicated in both cases of cure thus far.

1.4.2 An Overview of Antiretroviral Therapy

The advent of combination antiretroviral therapy (cART) has, to date, been one of the largest successes when addressing the HIV-1 epidemic. Most notably, individuals with access to cART experience less, if any, CD4 T cell depletion and subsequently do not transition into AIDS. As immune system function is relatively well-maintained in the presence of cART, these drugs prevent the onset of opportunistic infections which typically lead to AIDS-associated morbidity and death. Due to its acknowledged importance, the world health organization (WHO) has made antiretroviral therapy a cornerstone feature of their 90-90-90 initiative. This initiative aims for 90% of HIV-infected individuals to receive antiretroviral therapy and, of these individuals, for 90% to experience prolonged viral suppression. Currently, approximately 2/3rds of HIV-infected individuals are currently accessing these life-saving drugs.

Antiretroviral therapy functions through the inhibition of key steps within the HIV-1 life cycle. Specifically, antiretrovirals can be distinctly broken down into several classes: fusion inhibitors, co-receptor antagonists, reverse transcription inhibitors, integrase strand transfer inhibitors, and protease inhibitors. The combination of several drug classes can help prevent against drug-resistance in viral populations. To date, the FDA has approved 222 antiretroviral drugs for the treatment of HIV, including 23 combination-drug products.

1.4.2.1 Inhibitors of Viral Entry

Starting at the earliest stage of the viral life cycle are fusion inhibitors, which, as the name suggest, function through the disruption of viral machinery required for entry into an uninfected cell. Enfuvirtide (T20; Fuzeon, Roche) is a peptide mimetic that serves as a prototypic, FDA-approved fusion inhibitor. More specifically, this mimetic compound covalently interacts with the N-terminal region of HIV-1 gp41 to prevent pore formation at the surface of non-infected cells.[76] In addition to its usefulness in antiretroviral regimens, enfuvirtide provides utility in latency reversing assays designed to induce latent provirus or estimate reservoir size.[77,78] Due to its unique ability to stop viral entry, enfuvirtide helps prevent against the

14

overestimation of reservoir size, infected cell frequency, and latency reversing potential of therapeutic candidates.

In addition to fusion inhibitors, CCR5-receptor antagonists, such as maraviroc, are also capable of preventing viral entry into a host cell. The development of this drug class resulted from an observation that individuals with homozygous polymorphisms within CCR5 were resistant to infection by R5-tropic virus.[79,80] For maraviroc, function is conferred through its binding to the transmembrane cavity of CCR5, thus altering the receptor's conformation.[81] Due to its specific function as a CCR5 agonist, maraviroc-resistant HIV-1 populations have arisen which target the alternative, less commonly utilized co-receptor, CXCR4.[82] Additional efforts have identified mutations within the HIV constant region 4 (C4), such as N425K, that also convey resistance to maraviroc treatment.[83] Ultimately, this drug class can provide substantial benefit in the context of an antiviral therapeutic regimen, but information regarding viral tropism is essential.

1.4.2.2 Inhibitors of Reverse Transcription

As the seminal drug class for HIV-1 antiviral therapy, nucleoside reverse transcriptase inhibitors (NRTIs) are a cornerstone in most combinational regimens. NRTIs function through competitive inhibition of the reverse transcriptase enzyme and ultimately prevent chain elongation of DNA products.[84] The addition of purine and pyrimidine nucleosides is essential for the 3' elongation of the newly-developing DNA. An NRTI's function is analogous to that of other nucleosides but lacks a 3'-hydroxyl group required for chain elongation.[85] Therefore, incorporation of the nucleoside analogue terminates reverse transcription. As of 2016, clinical guidelines recommended that individuals on first-line antiretroviral therapy should take two NRTIs accompanied by a protease inhibitor (PI), non-nucleoside reverse transcriptase inhibitor (NNRTI), or an integrase strand transfer inhibitor (INSTI).[86] This is notable since NRTIs are less potent than alternative drug classes with more favourable inhibitory dose kinetics, including NNRTI and PI.[87] Despite this, eight NRTIs have become FDA-approved and several of them, such as emtricitabine (FTC) and tenofovir DF (TDF), are routinely incorporated into combinational therapies (**Figure 1-1**).

NNRTI-class drugs exhibit increased inhibitory capacity relative to NRTIs but similarly function to inhibit reverse transcription.[87] The virally-derived reverse transcriptase contains two subunits, p66 and p51. Rather than functioning directly on the active site of the enzyme, NNRTIs non-competitively bind to the p66 subunit to induce a conformational change that abrogates enzymatic function.[88] Importantly, these drugs are susceptible to variability within the reverse transcriptase gene as multiple individual mutations can alter or diminish NNRTI binding potential.[89]

1.4.2.3 Integrase Strand Transfer Inhibitors

Integrase strand transfer inhibitors (INSTIs) convey several advantages that make them important in many antiretroviral regimens. In a viral integration event, HIV-derived integrase transports proviral DNA to the host cell chromosome, which then incorporates via a 2-step catalytic reaction.[90] First, the proviral 3' end is processed in the host cell cytoplasm. This is subsequently followed by strand transfer and covalent linking of proviral DNA products to host cell DNA. As the name would suggest, INSTIs, such as dolutegravir or raltegravir, function through competitive inhibition of the strand transfer process by blocking the active site of integrase.[91] Interestingly, humans lack a homologue for HIV-derived integrase and, as a consequence, INSTIs are generally well-tolerated.[92] Like NNRTIs, resistance can arise from genetic mutations within the viral integrase gene. Despite this, INSTIs appear to be highly efficacious. In a large clinical study, raltegravir+abacavir(NRTI) achieved viral suppression of ~88% whereas Atripla (tenofovir, emtricitabine, efavirenz) only achieved 81% suppression at a 48-week follow-up.[93] This finding provides strong evidence that INSTI-based therapeutic regimens may replace, or provide an alternative to, current gold-standard antiretroviral therapies.

1.4.2.4 Protease Inhibitors

The viral protease is responsible for the maturation of viral particles via systematic cleavage of the gag and gag-pol polypeptides. This cleavage event results in the formation of protein products necessary for the proper assembly of new infectious virions. Protease inhibitors (PIs) function through the competitive inhibition of the HIV-1 protease enzyme, thus preventing the processing of viral polypeptides.[94] Importantly, protease inhibitors may also exhibit off-target

effects, such as altering the ability of dendritic cells to process and present peptide antigens.[95] Despite this caveat, eight different PIs have become FDA-approved since 1995. Each of these drugs, while having mechanistically similar or identical effects, each have relatively different pharmacokinetic profiles.[96]

1.4.2.5 The Shortcomings of ART

Antiretroviral therapy provides a substantial improvement to individuals affected by HIV-1 but, unfortunately, is not feasible as a permanent solution to the HIV-1 epidemic. One of the most striking characteristics of ART is the requirement to take it daily, for life. This inherent feature comes with several drawbacks. Firstly, providing cART to low- and middle-income countries is logistically difficult and expensive. In 2020 alone, UNAIDS estimates that the ongoing AIDS-response effort will cost ~$26.2 billion USD.[97] Secondly, individuals taking cART must ensure that they are in constant access of these drugs. 'Drug holidays', where an individual takes a break from therapy for an undetermined period, can lead to viral resistance and rebound viremia. Ultimately, ART-associated non-adherence is difficult to address due a diverse number of factors, including: lack of accessibility, drug-associated toxicity, patient-related drug/alcohol abuse, pill burden, and inconvenience.[98] Due to a large degree of interpatient variability, it is difficult, if not impossible, to address many of the shortcomings associated with a daily therapeutic regimen, such as ART. Therefore, finding alternatives that either facilitate, i) a reduced frequency of drug administration, or ii) increase ease of drug use, are of high priority. More modern drug formulations, such as Atripla, overcome some of these barriers by reducing pill burden and eliminating the confusing drug-timing intervals associated with older regimens.[99] This is accomplished by combining the three component drugs at clinically relevant dosages within a single capsule. Newer two-drug regimens are now under evaluation due to their potential to reduce ART-associated costs and toxicity.[100]

Despite a significant decrease in AIDS-associated illness and death, individuals on antiretroviral therapy still experience an increased risk of morbidity relative to the general population.[101,102] This includes an increased occurrence of myocardial infarctions, osteoporosis, and diabetes mellitus within cART-receiving cohorts.[103–105] In some instances, such as atherosclerosis in HIV-infected individuals, heightened inflammation and ongoing immune activation likely contribute

to the disease phenotype.[106,107] Such individuals experience heightened levels of secreted interleukin-6 (IL-6) and cell-expressed intercellular adhesion molecule 1 (ICAM-1), which are prevalent in instances of coronary blocking or stenosis.[107] Heightened immune activation in this cohort may be due to several mechanisms including, i) ongoing, low level viral replication, ii) viral protein production, or iii) ART-associated inflammatory lipids.[108–110] It is worth noting, however, that although immune activation is heighten relative to HIV-uninfected individuals, ART decreases immune activation relative to HIV-infected individuals lacking therapy.[111] Fortunately, as therapeutics continue to improve, drug-associated morbidity will continue to decrease. These therapeutic advances likely coincide with fewer people discontinuing ART due to drug-associated toxicity and contribute to an overall improvement to quality-of-life.

1.4.3 Limiting Establishment – Initiation of cART During Primary Infection

Upon initiation of cART, a small subset of HIV-infected lymphocytes revert to a low metabolic state, trapping HIV-1 provirus within the cell. It is theorized that early initiation of cART may limit the size of the quiescent proviral reservoir, and thus limit the degree of viral recrudescence post-treatment cessation. Furthermore, cART initiation during primary infection may help preserve immune responsiveness and prevent chronic immune activation.[112] In the instance of the Mississippi Child, a baby born to an HIV-positive mother was at high risk of HIV-infection, and subsequently initiated cART 30 hours after birth.[113] If infection did, in fact, occur, the pool of virus capable of establishing a long-lived reservoir would be minimal. Treatment continued up until 18 months of age, after which treatment was interrupted. Analysis of plasma HIV RNA and proviral DNA at 30 months remained negative, providing evidence that early treatment initiation may provide long-lasting protection against infection.[113] Although plasma viremia remained undetectable at a 21.9 month follow-up, viral loads rebounded to 16,750 copies/mL by 27.6 months and cART was immediately re-initiated.[114] Despite requiring re-administration of cART, the Mississippi child furthered our understanding of HIV-1 infection and establishment. For instance, the timeline for HIV-1 establishment outpaces rapid administration of cART and limits the opportunity to utilize post-exposure programmes as a reliable method for HIV prevention. However, in such instances, early initiation of cART may contribute to long-term remission post-therapy interruption. These findings, in summation, suggest that early access to cART may

substantially limit, but not prevent, HIV-1 reservoir establishment while preserving immune function. This finding is further supported by findings in the VISCONTI cohort, which involves fourteen (14) post-treatment controllers who initiated cART early after primary infection, and maintained prolonged control of viremia upon treatment cessation.[112] The study concluded that early treatment may permit up to 15% of individuals to maintain viral control for greater than 24 months, which is substantially higher than individuals treated later during infection.

1.5 Achieving HIV-1 Latency Reversal
1.5.1 The Shock and Kill Strategy for HIV-1 Eradication

The latent reservoir likely establishes during a change in metabolic status, wherein HIV infects a cell transitioning from an effector to a memory subset. As shown in a macaque model, the formation of viral reservoirs within the host CD4 T cell population can occur within the initial days of infection.[115] Early ART initiation, despite minimizing the reservoir size, is incapable of preventing its establishment.[116,117] Notwithstanding, ART represents a crucial timepoint, as majority of the replication competent reservoir is seeded around the time of cART initiation.[118] Due to the inability to access cell host machinery, integrated provirus is trapped in a state of dormancy devoid of RNA and protein production. The lack of transcription factors, prevalence of epigenetic modifications, and impaired nuclear export may all contribute to this latent cellular phenotype.[119,120] Because these cells do not produce viral transcriptional products, current therapeutic strategies are unable to effectively target these cells for removal. Furthermore, it is unlikely that the reservoir will extinguish spontaneously due to its long half-life of approximately 44 months.[121] It is because of this persistent pool of provirus that cART remains necessary for life – it prevents bystander cell infection from reservoir-derived virus. Conversely, in the presence of cART cessation, latently infected cells can initiate transcription/translation of new viral particles to establish *de novo* infection within its cellular targets. Despite the tremendous contribution of cART to inhibit progression from HIV to AIDS, cART is not a sustainable answer. This is because of cART-induced toxicity and immune dysregulation within the patient, and the lack of resources required for cART to be a long-term

solution. Therefore, it is pertinent that we investigate strategies that contribute to a disease-free status.

The most commonly investigated approach for removing the latent reservoir is the 'shock-and-kill' strategy for HIV-1 eradication. In this strategy, HIV-infected cells are 'shocked' into a state of transcriptional activity using a latency reversing agent (LRA). Upon reactivation, the production of viral RNA and protein facilitates recognition and killing via immune-mediated or viral cytotoxic effects (**Figure 1-3**). This process is contingent on maintained anti-retroviral therapy to prevent the establishment of new reservoirs, as mentioned previously. Many different LRAs have been investigated for their potential ability to induce HIV-1 transcriptional reactivation, many of which are described below.
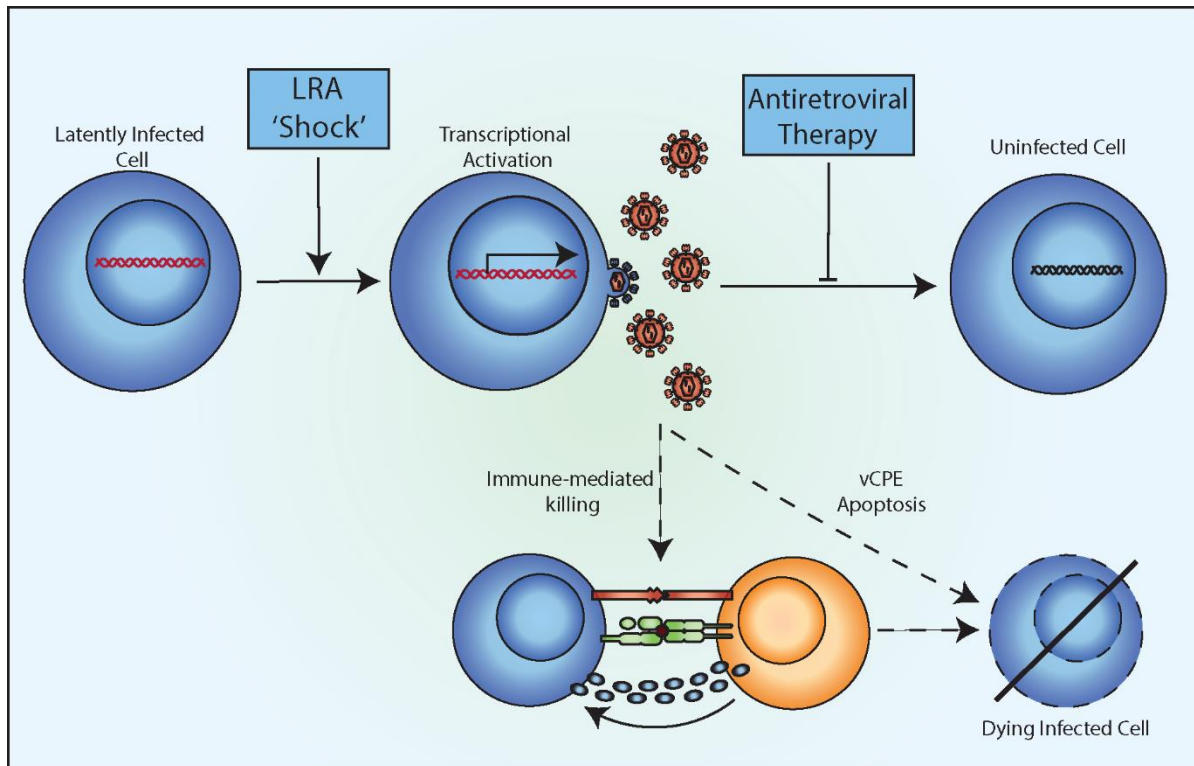
Figure 1-3: The shock-and-kill method for HIV-1 transcriptional reactivation
Latently infected cells are targeted for reactivation by using a latency reversing agent (LRA), or 'shock', to re-initiate transcription. Transcriptionally active virus produce viral proteins and virions which lead to infected host cell death via i) immune mediated killing, ii) viral cytopathic effects (vCPE) and/or, iii) apoptosis. Progeny virions are unable to infect bystander cells due to the presence of antiretroviral inhibition.

1.5.2 Histone Acetylation

The acetylation status of the HIV-1 LTR regulates viral transcriptional activity and, therefore, modulates viral latency. Modification of the LTR occurs through the enzymatic function of either histone acetyltransferases or histone deacetylases (HDAC), which promote or suppress transcription, respectively **(Figure 1-4)**.[122–124] Histone acetyltransferases, which are antagonized by HDAC, open the chromatin conformation and can promote complexes essential for transcriptional activity. This is accomplished by the remodeling and hyperacetylation of nucleosome-0 (nuc-0) and nucleosome-1 (nuc-1) within the 5' LTR, which act as 'switches' to initiate virus production.[125] The opposing HDACs alternatively promote a heterochromatic environment and transcriptional downregulation via the removal of acetyl groups from lysine residues on histone tails, thus causing chromatin compaction.[126,127] Downregulation is accomplished both directly and indirectly through i) deacetylation at HIV-integrated sites and, ii) modification of transcription factors, such as NF-κB p50 subunit.[77,128] The continual interplay between these enzymes ultimately dictates cellular chromatin conformation and are worth investigating as potential targets to induce transcriptional upregulation.

In order to create a favourable environment for cellular transcription, histone deacetylase inhibitors (HDACis) have been extensively studied for their ability to induce latency reversal and have formed the basis of numerous clinical trials worldwide. HDACi prevent silencing of the HIV-1 proviral genome through direct inhibition of the enzyme, histone deacetylase, thus facilitating hyperacetylation and remodelling to an open chromatin formation.[129]

This family of small molecule inhibitors have been targets of investigation for their potential role as latency reversal agents , with valproic acid (VA) becoming the first HDACi to enter clinical trials.[78] Despite its high tolerance, chronic VA therapy did not significantly decrease the detection of latently infected cells when compared to patients on prolonged cART alone.[130] The first 'next-generation' HDACi, suberoylanilide hydroxamic acid (SAHA, Vorinostat), was initially approved for the treatment of T cell lymphoma before being investigated for its potential role as an LRA.[131] In 2012, Archin et al. described the single-dose effects of Vorinostat in eight patients, following proof-of-concept studies identifying latency disruption *in vitro*.[129,132–134] The drug's bioactivity was determined using measurements of histone acetylation *in vivo*. Archin demonstrated a mean

4.8-fold increase in the amount of HIV-1 RNA being produced six hours post infusion with a single dose of VOR. Following a multiple dose regimen, VOR maintained high tolerance, although cell-associated HIV RNA stimulation was greatly stunted following the initial dose, potentially resulting from a drug related refractory period.[135] Of importance, new findings may suggest SAHA increases the susceptibility of target cells to HIV-1 infection.[136]

The chemotherapy drug, Panobinostat, has also been implicated as a potential LRA. Fifteen individuals on suppressive ART received oral Panobinostat three times weekly for eight weeks, with cell-associated viral RNA measured at each time point.[137] The mean maximum increase in HIV-1 RNA associated with the Panobinostat treatment regimen was 3.5-fold (range 2.1-14.4), showing latency disruption *in vivo*, but failed to reduce the detection of latently infected cells. Nine individuals partook in treatment interruption, with mean viral rebound occurring within 17 days. Only grade I adverse events were noted, indicating high tolerance for Panobinostat.

The HDACi, Romidepsin, disrupts latency in HIV-1 infected cells and has shown greater potency than Vorinostat and Panobinostat *in vitro*.[138,139] Consequently, Søgaard et al conducted a clinical study in which six patients on suppressive ART received weekly injections of Romidepsin (5mg/m^2) intravenously for three weeks.[140] On average, HIV-1 transcription increased nearly four-fold from baseline levels when observing levels of unspliced, cell-associated HIV-1 RNA. In five out of six individuals, plasma HIV-1 RNA became readily detectable compared to baseline levels of <20 copies/mL. Infusions of Romidepsin did not decrease detection of HIV-1 specific CD4+ T cells *in vivo*.

Despite the numerous trials at the clinical and pre-clinical stages of development, none of these treatments can clear the infected cells. Clearly, a disparity exists between the *in vitro* and *in vivo* models. It would appear unusual that, despite *in vitro* success, the models fail to make any noticeable difference in viral clearance. To amend this conflict, several different hypotheses have been formed. First, certain HIV-specific cell lines over-express Bcl-2, a protein responsible for cell survival. As a result of this over-expression, cells do not undergo viral cytolysis in the presence of HDACis.[141] Additionally, the function of cytotoxic cells varies between *in vitro* and *in vivo* models, thus altering the ability to 'kill' latently-infected cells. Whereas *in vitro* studies of latency reversal

report abrogated CD8+ and NK cell function in response to HDACi treatment, no such circumstances occurred in clinical trials for the same drugs.[140,142,143] Although HDACis may induce the production of viral RNAs and proteins from latently-infected cells, such defects in clearance would prove detrimental to this approach. Finally, in the case of chronic infection, cells are likely to exhibit a high degree of T cell exhaustion and may, as a consequence, impair the clearance of infected cells.[144] Because multiple mechanisms exists wherein latently-infected cells can persist post-reactivation, the success of an HDACi regimen would be contingent upon co-treatment with a mechanism to facilitate 'kill'.
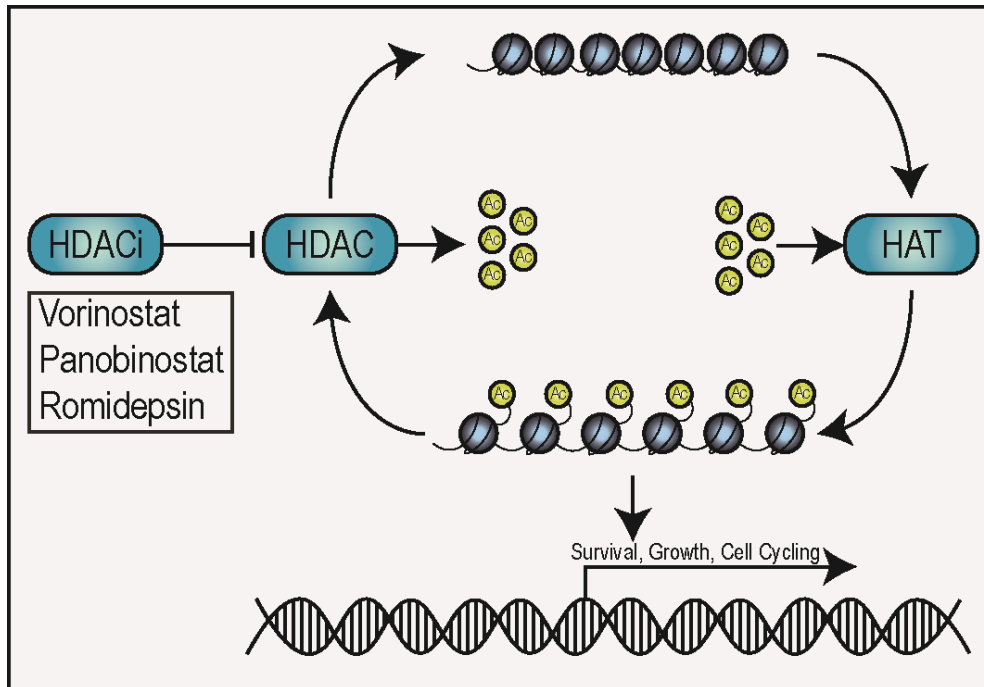
Figure 1-4: Mechanism of the latency-reversing histone deacetylase inhibitor

Histone deacetylase inhibitors (HDACi), such as Vorinostat, Panobinostat, and Romidepsin, function through direct inhibition of the histone deacetylase (HDAC) enzyme. HDAC are capable of stripping acetyl groups from chromatin, facilitating a shift from euchromatin to heterochromatin. Histone acetyltransferases (HAT) add acetyl groups to chromatin, preventing tight compaction and creating a euchromatic environment. Euchromatic environments are transcriptionally favourable, thus leading to the production of mRNAs and downstream protein production.

1.5.3 BET Bromodomain Inhibitors

The bromodomain protein Brd4 is a major barrier to HIV-1 latency reactivation through its competitive inhibition of the Tat-SEC interaction.[145] JQ1 is a bromo and extra terminal (BET) bromodomain inhibitor capable of Brd4 inhibition and induction of HIV-1 expression in latently infected cell lines.[145,146] Interestingly, in the absence of Tat, JQ1 is also the only latency reversal agent capable of inducing HIV mRNA splicing.[147] The selective small molecule is further involved in the downregulation of T cell transcriptional genes, while upregulating genes responsible for histone modification.[148] An *ex vivo* investigation using CD8+-depleted PBMCs from patients on suppressive ART identified that combination JQ1+Bryostatin-1 administered at clinically relevant doses stimulated reactivation at the same frequency as the anti-CD3/CD28 positive control, and was supported with findings by Laird et al earlier in 2015.[146,149] However, the findings of these studies are confounded by the results of another *ex vivo* study that failed to observe any significant impact on latency reversal when JQ1 was administered alone.

1.5.4 Protein Kinase C Agonists

Bryostatin-1 is a macrocyclic lactone that functions as an agonist to protein kinase C (PKC).[150] In an *ex vivo* study of common LRAs, Bryostatin-1 was the only agent shown to effectively reverse HIV-1 latency *in vivo*.[151] However, the effect of Bryostatin administration was only 4% when compared to maximal reactivation via T cell activation, emphasizing a need for combinational drug approaches to overcome the multiple mechanisms dictating latency *in vivo*.[149] This PKC agonist, when combined with HDACis or bromodomain inhibitors, was capable of potent re-activation of HIV-1 transcription.[21] Regarding toxicity, a PI clinical trial reported high tolerance to single-dose Bryostatin-1 administration in patients on suppressive ART receiving 10ug/m^2 or 20ug/m^2.[152] Unfortunately, the plausibility of such studies will likely be limited due to reports of grade III/IV adverse effects associated with Bryostatin-1 administration at higher doses, as seen in clinical trials for various cancer treatments.

Prostratin has also received attention due to its ability to stimulate HIV-1 transcriptional activity whilst preventing *de novo* infection.[153] Like Bryostatin-1, Prostratin is a potent stimulator of NF-kappaB and consequentially activates the HIV-1 LTR, leading to the viral transcription of latent provirus.[122] Prostratin was shown to be one of three LRAs (including Romidepsin and Bryostatin-

1) that induced significant increases in viral mRNA (mean 7.7-fold increase) when administered alone.[151] Furthermore, intracellular HIV-1 mRNA levels increased further when Prostratin was combined with several LRAs, specifically Romidepsin or JQ1, exemplifying the synergistic nature of the molecule.[151]

Unfortunately, the natural availability of Bryostatin-1 and Prostratin is low, and demand an alternative means of acquirement. The development of mechanistically identical synthetic analogues has been used to overcome this obstacle.[154,155] Surprisingly, the PKC-binding potential of the Prostratin analogue is up to 100-fold greater than its naturally occurring counterpart when observed *in vitro* and *ex vivo*.[154] The Bryostatin-1 analogue also shows ≈1000-fold increase in the induction of latent HIV-1 when compared to Prostratin *in vitro*.[155] However, neither Prostratin nor its synthetic analogue have been tested in humans to determine its safety or efficacy. Nevertheless, it may be possible to cautiously infer these consequences *in vivo* based on the mechanistic similarities between Prostratin and Bryostatin-1.

1.5.6 Virus-Like Particles

The development of virus-like particle (VLP) formulations provides a powerful alternative to contemporary vaccine regimens. More specifically, VLPs deliver an efficacious and flexible vaccine platform that, importantly, ensures a high degree of safety due to the innate inability for the VLP to replicate.[156] This is due to the absence of a viral genome, which is present in viral particle (VP) formulations but absent in VLPs. In the context of HIV-1, virus-specific mutations are also incorporated to ensure safety, including the lack of a functional packaging sequence and absence of the 5' LTR.[157] Virus-like particles traditionally self-assemble with the provision of core envelope or capsid proteins and, in the case of HIV-1, only requires Gag protein for immature particle assembly.[158,159] Despite this, HIV-1 VLPs benefit from the addition of all structural proteins, as they can contribute to heightened antigenicity. By ensuring the presence of all viral proteins, a VLP can appear morphologically similar, if not identical, to its wild-type counterparts.[160] This includes the presence of identical surface proteins and particle size. Production methods can widely vary, with VLP production occurring in plants, yeast, and bacterial/insect/mammalian cell lines.[161–163]

Virus-like particles express T cell epitopes, which can activate different T cell subsets, including Type-1 T helper cells (T$_H$1) and CTLs.[164] The subsequent functioning of these cells is implicated in pro-inflammation, cytokine secretion, cell killing, and APC activation. As an exogenous antigen, VLP formulations primarily utilize major histocompatibility complex II (MHC-II) to signal T helper cells in an MHC-restricted manner. As MHC:TCR interactions are highly specific, this provides a tool for the targeting of virus-specific T cells.[165] Moreover, studies have also shown that VLPS can cross-present to MHC-I through a TAP-independent endosomal pathway, thus simultaneously stimulating CTL activation.[166,167] The CTL/T$_H$1-stimulatory effect of VLPs is further complemented through the incorporation of various stimuli. In one such instance, incorporation of TLR7 and TLR9 agonists provided a boosting effect to a norovirus VLP vaccine formulation.[168] The ability to adjuvant VLP-based vaccine regimens provides additional avenues for investigation and may drastically impact vaccine efficacy.

In addition to their ability to efficiently target T cells for activation, VLPs can also contribute to the induction of B cell responses. This can be through the induction of complement-dependent cytotoxicity, T cell regulation, and antibody-dependent cellular cytotoxicity (ADCC).[164] Of notable importance, VLPs exhibit a high degree of organization and display geometric pathogen associated structural patterns (PASP) on their surface.[158,164] Such patterns may efficiently cross-link BCRs, which can heightened B cell induction.[169] In the context of HIV-1, increasing the prevalence of envelope proteins on the cell surface may contribute to improving B cell responsiveness. In one such study, a 10-fold increase in surface envelope glycoprotein spikes significantly upregulated Env-specific B cell responsiveness.[170] Alternatively, peptides and proteins can be linked to the surface of the VLP without significantly altering the size of the particle.[171,172] In all such instances, B cell activation will ultimately induce antibody production, which can be used to specifically target virally-derived antigens. By modifying the antigens present on the surface of a VLP, it becomes possible to create a highly tailored antibody response to a region of interest. Moreover, by adjuvanting virus-like particles with stimuli, such as TLR7/8 RNA, it is possible to drive isotype switching to IgG.[173]

Currently, little is known about the efficacy of a virus-like particle formulation as a vaccine for HIV-1 prophylaxis or cure. Due to high mutation rates within the HIV-1 genome, a virus-like

particle formulation would need to present a disperse array of peptides to ensure optimal presentation via the MHC:TCR complex. In such a way, it is possible to target HIV-specific T_h1 cells for activation which, coincidentally, also serve as the primary reservoir for quiescent provirus. However, due to the low surface expression of envelope trimers on wild-type HIV-1 and, by extension, native VLPs, it is unlikely that BCRs will efficiently cross-link, thus limiting the production of protective antibodies. As such, VLP-mediated antibody responses may require that the VLP express more viral proteins on its surface.

Although the use of virus-like particles for HIV-1 curative therapy is relatively novel, an increasing amount of evidence suggests that HIV-1 VLPs would be effective latency reversing agents. Originally, researchers found that HIV-1 preferentially infects HIV-specific CD4 T cells over CD4 T cells with alternative TCR specificities.[174,175] This finding was compounded by studies revealing that latency reversal was efficiently achieved through TCR stimulation using HIV-derived peptides.[176,177] Therefore, VLPs targeted to MDDC for presentation to HIV-specific T cells represents an exciting avenue for latency reversal research.

1.6 Adaptive Immunity and HIV-1

1.6.1 Dendritic Cells – Characteristics and Functions

Dendritic cells have a critical role as an intermediary between the innate and adaptive immune systems. These cells are capable of sensing pathogen-/danger-associated molecular patterns (PAMPS/DAMPS) to induce acute inflammation and mediate adaptive responsiveness in the context of antigen processing and presentation.[178] Typically, immature dendritic cells (iDCs) provide key surveying and phagocytic functions, whereas mature DCs mediate antigen processing and presentation in the context of MHC-I/II. Maturation is also accompanied by up-regulation of the co-stimulatory molecules CD80 and CD86, which contribute to the activation of T lymphocytes.[179] There are three primary DC subsets, including: plasmacytoid DCs (pDCs), conventional/myeloid DC1 (cDC1s), and DC2s. Of importance, DC1 cells are known as such due to their ability to drive naïve T cell differentiation into Th_1 T cells in a cytokine-independent process.[180] This is important because, during natural infection with HIV-1, CD4+ T_h1 cells are the primary target for viral infection. In the context of the work herein, we focus on monocyte-derived dendritic cells (MDDCs), which are routinely used *in vitro* due to their similarities with

conventional myeloid-derived DC1s, including morphology and function.[179] By contrast, MDDCs are not typically present in steady state, but are more readily produced in pro-inflammatory environments.[181,182] The MDDC subtype is further differentiated by the expression of DC-SIGN/CD209a on the cell surface.[182] These cells are readily differentiated from monocytes in the presence of IL-4 and GM-CSF, making them a useful for studies of dendritic cell function.

1.6.2 Dendritic Cells in HIV-1 Processing

Dendritic cells typically utilize two pathways for antigen processing, one for exogenous antigens and the other for endogenous antigens. In the context of HIV-1 viral particles, uptake will occur via the exogenous pathway **(Figure 1-5)**. Typically, dendritic cells begin uptake through either receptor-mediated endocytosis or, less often, pinocytosis. Upon internalization, the HIV particle/antigen is degraded into peptides through a series of endocytic compartments of sequentially increasing acidity. Degradation is first initiated within the early endosome (pH ~6.0-6.5) before transitioning to the late endosome (pH ~4.5-5.0), and ultimately the endolysosome (pH 4.5). During this time, antigen is processed into peptides ranging from 13-18 residues, which is ideal for binding to MHC II within the MHC-II-containing endosomal compartments (MIIC). Importantly, this processing pathway is dependent on i) hydrolytic enzymes within endosomal compartments and, ii) low pH. As a consequence, this process can be disrupted through inhibitors of protease activity (e.g. Pepstatin A) or through increasing the pH within endosomal compartments (e.g. chloroquine).[95,183]

Before MHC-II can interact with peptides within the MIIC, the molecules must first be synthesized and transported. MHC-II αβ chains are synthesized within the rough endoplasmic reticulum (RER), as are MHC-I molecules from the endogenous pathway. In order to shuttle out of the RER, MHC-II molecules must interact with the invariant chain, CD74, which interacts with the peptide binding groove to prevent MHC-II molecules from interacting with peptides destined for MHC-1 presentation. Upon MHC-II:CD74 engagement, the complex shuttles through the golgi complex and into the endocytic compartment. As proteolytic activity increases, the invariant chain is cleaved except for the class-II associated invariant chain peptide (CLIP). CLIP remains within the MHC-II peptide-binding pocket, and prevents pre-mature binding. HLA-DM catalyzes the response which removes the CLIP in exchange for

antigenic peptides. The MHC-II:peptide complex is shuttled to the plasma membrane, where it can interact with lymphocytes in a MHC-restricted context.

Dendritic cells have become an increasingly desirable strategy for many vaccine regimens due to their ability to present antigen peptides in a highly specific context. By pulsing dendritic cells with a specific antigen, we can mediate MHC-II presentation to activate particular subsets of CD4$^+$ T cells. In the context of latency reversal, this provides a strategy to specifically target T cells expressing HIV-specific TCRs.
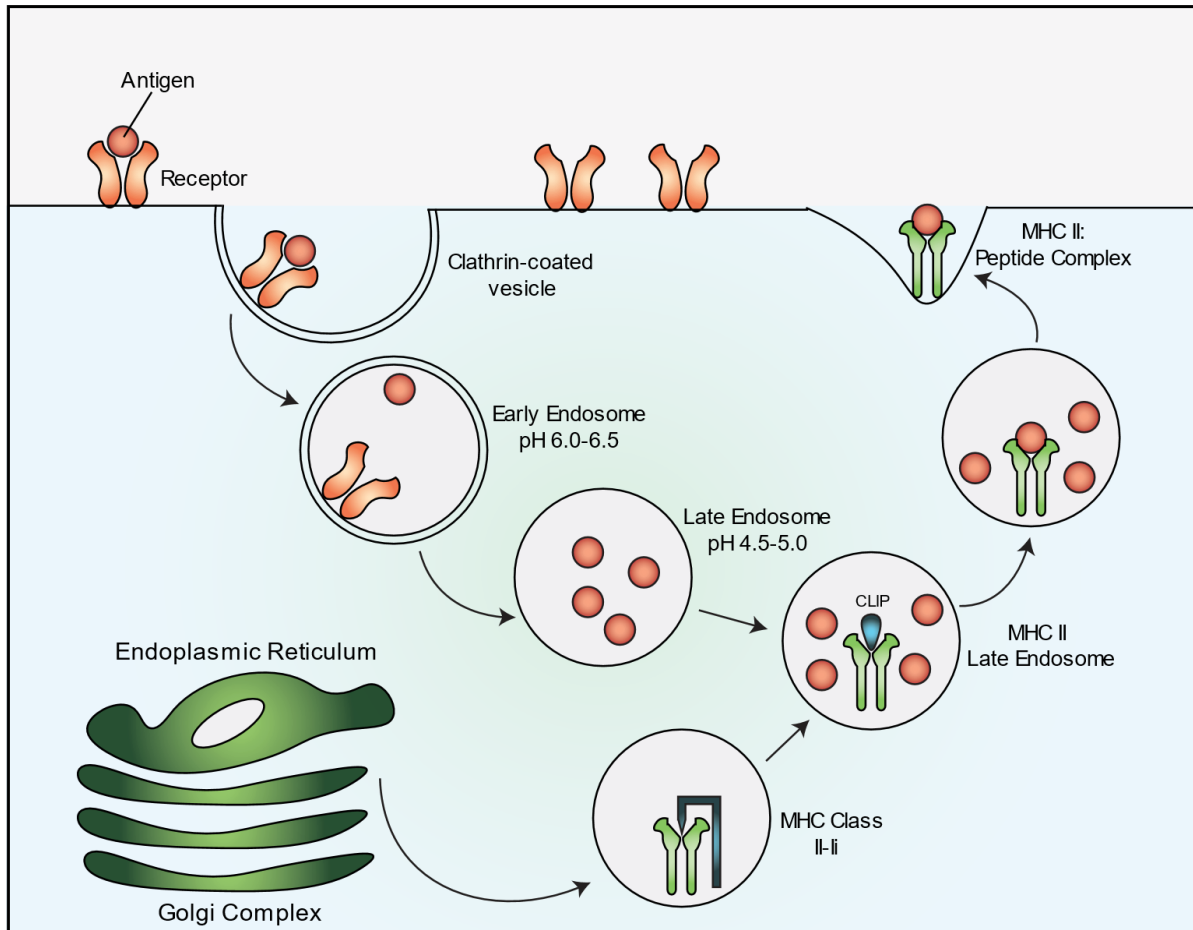
Figure 1-5: The exogenous dendritic cell processing pathway.

Endocytosis occurs via internalization of antigen-bound receptor molecules via clathrin-coated vesicles. The pH of the endosome decreases, allowing for antigen processing into smaller fragments. MHC:II molecules are produced in the endoplasmic reticulum and shuttled through the Golgi complex. The invariant chain (Ii) is cleaved, leaving CLIP in the receptor binding site of the newly formed MHC II molecule. CLIP is replaced with processed peptide before the complex is shuttled to the surface of the dendritic cell for presentation. Ii, invariant chain; MHC, major histocompatibility complex.

1.6.3 T Cells – Characteristics and Function

T cells are one of the functional cornerstones of adaptive immunity, with roles in immune mediation, homeostasis, and immunological memory. Furthermore, these cells have highly diverse TCRs and can detect up to 100 million different specificities.[184] T cells are also a necessary component that mediate immune tolerance. As such, T cells are a vitally integrated part of human immunity, and dysfunction of these cells can lead to autoimmune disease or pathogen susceptibility.

In the beginning, hematopoietic stem cells (HSCs) differentiate into multipotent progenitor cells that follow either the myeloid or the lymphoid lineage. These cells arise within the bone marrow and, in the case of T lymphocyte development, migrate to the thymus to undergo maturation and selection. Knowledge of the underlying process, referred to as thymopoiesis, has largely been investigated in mice due to the ability to perform thymectomies and bone marrow reconstitutions.[185] In the thymus, CD4$^-$CD8$^-$ progenitor cells experience T cell receptor (TCR) rearrangement to yield double positive (DP) thymocytes.[186] DP thymocytes subsequently engage in positive selection, wherein the CD4$^+$CD8$^+$ cells are subjected to MHC-I and MHC-II class molecules.[187] During positive selection, cells can either i) fail to bind MHC-I/II and undergo apoptosis, ii) bind MHC-I and mature into a CD8$^+$ T cell or, iii) bind MHC-II and mature into a CD4$^+$ T cell. Positive selection permits the survival of any T cell capable of binding MHC, irrespective of binding affinity. This process ultimately yields single-positive thymocytes that upregulate the CCR7 chemokine for migration to the medulla. In this region, negative selection permits the removal of high affinity, self-reactive clones. Interestingly, the thymus is largest at birth and experiences changes to volume, epithelial density, and fat content over time.[188] It is expected that thymopoiesis begins to significantly decrease at ~40 years old based on studies investigating DP thymocyte prevalence within the thymus.[189]

In the periphery, naïve T cells undergo effector and memory differentiation in a triphasic process. First, clonal expansion occurs in response to TCR-mediated antigen recognition, followed by the contraction phase, during which most effector cells die via apoptosis and, finally, the memory phase, which contributes to primed immune reactiveness in response to repeat infection.[190,191] In studies evaluating CD8$^+$ memory cell persistence, it was found that

approximately 5-6% of effector cells transitioned to a memory phenotype and were detectable at 25 years post-vaccination.[192,193] Such studies have been recapitulated across a multitude of acute viral infections, all of which exhibited similarities in T cell response kinetics and magnitude.[186]

Of notable importance are CD4^{+} T cells that engaged with MHC-II during thymic selection. These cells, known as T helper (T_h) cells, can be stratified into several distinct groups: T_H1, T_H2, T_H17, and T_{FH} cells.

1.6.4 T Cells in HIV-1 Pathogenesis

HIV-1 is heavily implicated in the depletion of CD4^{+} T cells over the time-course of infection. This depletion ultimately compromises adaptive immunity and leads to the onset of acquired immunodeficiency syndrome (AIDS). During AIDS, the body is more susceptible to co-infection with opportunistic pathogens, which depend on an abrogated adaptive immune response to establish and disseminate infection. As such, although HIV-1 weakens adaptive immunity through T cell destruction, it is often the opportunistic infection that directly contributes to severe morbidity or mortality. Studies of how CD4^{+} depletion occurs may shed light on potential avenues to reduce T cell depletion. It is important to note, however, that sustained cART therapy can maintain relatively stable T cell numbers.

Unsurprisingly, many studies have contributed T cell decline to apoptosis in both the productively-infected and bystander CD4^{+} or CD8^{+} T cell cohorts.[194–196] Early investigations into the cause of T cell decline, specifically within the bystander population, identified antigen-presenting cells, such as monocyte-derived macrophages, as key players in indirect apoptosis.[195] Other mediators of apoptosis, such as Fas, a receptor for cell death signaling, are upregulated in the CD4^{+} and CD8^{+} populations within HIV-infected individuals.[197,198] This correlates with increased FasL expression in cells such as, natural killer (NK) cells, macrophages, and monocytes.[198] Despite this finding, it is worth noting that HIV-infected cells expressing Fas are not the predominant cells succumbing to apoptosis *in vivo*, and many apoptotic cells from infected patients lack Fas expression.[199,200] Fas:FasL interactions may also contribute to apoptosis during the contraction phase of infection, when expanded populations undergo activation induced cell death (AICD).[201] Interestingly, more recent investigations have identified

decreased thymic output of T cells in response to HIV-1 infection, likely resulting from i) direct viral cytopathic effects and ii) increased levels of indirect apoptosis within the immature thymocyte population.[201] This decrease in T cell output may subsequently contribute to the overall loss of circulating T cells detected in the blood and atrophy of the thymus. A good indicator of this phenomenon is the number of T-cell receptor rearrangement excision circles present in circulating cells, which is inversely correlated with HIV-1 viral load.[202]

Viral proteins also contribute to pathogenesis within HIV-infected individuals. For instance, upon binding of envelope gp120 protein, cellular apoptosis is initiated through either, i) Fas upregulation and engagement, ii) upregulation of TRAIL death receptors 4/5 (DR4/5), or iii) upregulation of proapoptotic transcription factors, such as NF-kB and p53.[203–205] The relative contribution to each of these pathways in the context of gp120:CD4$^+$ engagement remains unknown, however, it is important to appreciate the polyfunctional nature of gp120 pathogenicity. Conversely, Tat protein functions through its production and secretion within infected cells, and can subsequently enter uninfected cells via clathrin-mediated endocytosis.[206] Additionally, studies utilizing exogenous tat on monocytes and macrophages have shown TRAIL upregulation and apoptosis of bystander cell populations.[207] Another viral protein, nef, can similarly upregulate expression of Fas on CD4$^+$ T cells.[208] Additionally, nef contributes to the heightened expression of programmed death 1 (PD-1) which, as its name implies, modulates CD4$^+$ T cell survival.[209] Nef is further capable of entering uninfected cells via the secretion of endosomes from HIV-infected CD4$^+$ T cells, and likely facilitates bystander cell death.[210] Interestingly, viral Vpr exhibits antiapoptotic characteristics until after G(2) arrest, after which Vpr can mediate the release of cytochrome c and elicit caspase 3/9 function.[211,212] It is evident that HIV-1 has several mechanisms which can kill CD4$^+$ T cells, both directly and indirectly. This can be problematic in the case of uncontrolled infection; however, the advent of antiretroviral therapy can regulate the pathogenicity of HIV-mediated apoptotic mechanisms.

More recent studies suggest that CD4$^+$ T cell depletion may not be fully attributable to apoptosis. In fact, estimates suggest that only 5% of virally-mediated cellular depletion is due to apoptosis, while the remaining 95% results from pyroptotic events.[213] This claim can be substantiated upon closer analysis of CD4$^+$ T cell death and, specifically, by looking at the

necessary steps of the viral infectious cycle required to mediate death. Through such investigative efforts, it was shown that bystander cells receiving entry inhibitors (AMD3100) or fusion inhibitors (T20) could withstand death while, conversely, nucleoside reverse transcriptase inhibitors (NRTIs) and inhibitors of upstream processes did not protect cells from destruction.[214] As an exception, NNRTIs, which directly inhibit the function of reverse transcriptase, could protect against cell death. This indicates that some form of transcriptional elongation must be necessary to initiate a death response.[215] Evidently, there is a period of time after entry but before integration wherein the necessary components for cellular death occur. Specifically, CD4+ T cell loss is likely attributed to non-productive HIV-1 infection within resting cells.[214,216] To address this phenomenon, Monroe et al. performed studies using affinity chromatrography and mass spectrometry to identify a cellular censor and ultimately identified IFI16.[217] The IFI16 protein is capable of detecting the accumulation of viral DNA fragments within the cell cytoplasm and, upon reaching a threshold concentration, forms an inflammasome to initiate caspase-1 mediated pyroptosis.[213] shRNA-induced knockdown of the inflammasome or caspase-1 protein both prevented cell death within abortively infected cells, whereas caspase-3 inhibition was non-protective. In summation, a majority of CD4+ T cell loss during HIV infection is likely due to abortive infection of resting and bystander cells with low transcriptional activity, wherein the accumulation of viral DNA fragments triggers a pro-pyroptotic response.

1.6.5 T Cell Signalling

TCR signalling is initiated when the TCR engages with a peptide-MHC-II complex presented by an antigen presenting cell, such as an MDDC. The TCR, itself, is sufficient for recognition and engagement of the peptide:MHC complex, however, signalling requires the ζ chain and CD3 molecules due to the 'immunoreceptor tyrosine-based activation motifs' (ITAMs) present within their cytoplasmic domains (**Figure 1-6**).[218] The co-localization of the CD4 coreceptor is sufficient to recruit Lck, which is responsible for phosphorylation of tyrosine residues within the ITAMs. Phosphorylated residues can interact with the Zap70 molecule via the SH2 region. Zap70 engagement on the cell surface is sufficient for its activation, which is stabilized by phosphorylation and is responsible for 'linker of activation of T cell' (LAT) activation.[219] These

sites of phosphorylation are responsible for i) calcium provision for Ras/MAPK pathway activation and, ii) Grb2/Gads recruitment for downstream Ras/Rac/Rho GTPase activation.[220] At the cellular membrane, interactions with CD28 and the TCR cause concurrent downstream signalling. CD28 activates PI3K and, through a series of phosphorylation events, induces the PIP_2/PIP_3/ITK kinase/PLC1 cascade.[221] PLC1 subsequently generates the DAG and IP_3 substrates. DAG interacts with protein kinase C (PKC) to activate Ras. Alternatively, IP_3, upon binding its cognate receptor, is responsible for Ca^{2+} release and is implicated in the production of transcription factors, such as NFAT. These concurrent signaling pathways ultimately contribute to T cell fate in regards to proliferative ability, transcriptional activity, and cell survival. Inhibition across multiple pathways can contribute to a highly abrogated T cell expression phenotype.

Understanding the metabolic tendencies of T cells throughout HIV infection is also necessary when attempting to understand viral latency and re-activation. Following TCR stimulation, the metabolic transport protein, Glut1, is upregulated via the Myc protein.[222] The degree of cellular metabolism further drives T cell activity *in vivo*. Increases in glucose uptake and glycolysis, determined through experiments involving the transporter protein Glut1, reveal that glucose metabolism contributes to cellular proliferation, survival, and cytokine production.[222] T cell metabolism can be further complemented with CD28 co-stimulation, which subsequently activates the mTOR pathway and mediates surface localization of Glut1.[223] The inability of an effector T cell to upregulate glucose metabolism is implicated in cellular inactivity and promotion of cell death.[224] Cells that survive are prone to anergy, as are cells exhibiting deficiencies in mTOR signalling.[225,226] Therefore, latency reversing strategies designed to target the latent reservoir must also consider the metabolic state of the cell, as unfavourable conditions will surely hinder transcriptional reactivity.
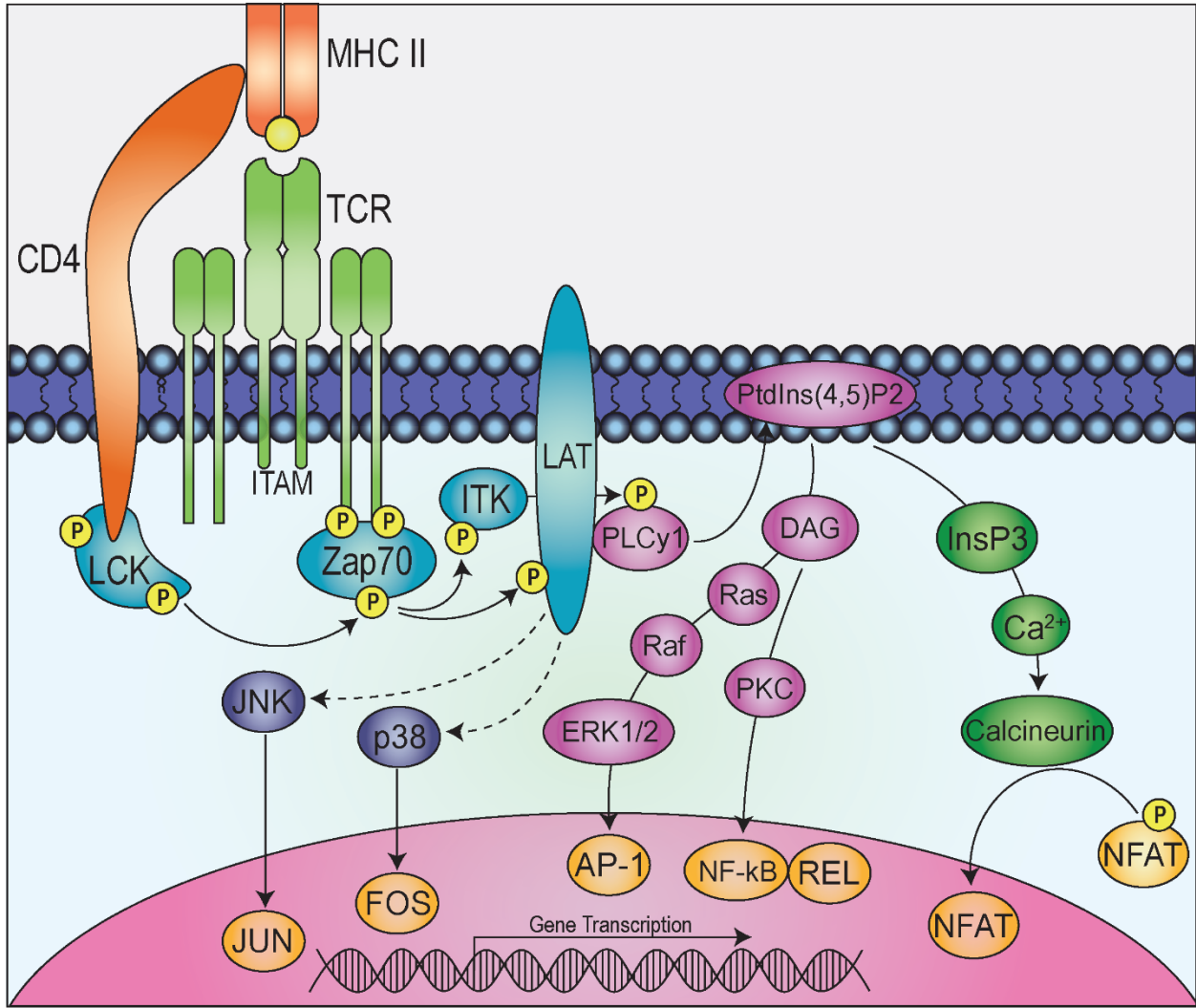
Figure 1-6: A schematic of T cell signalling through the T cell receptor
MHC:peptide interaction with the T cell receptor (TCR) initiates T cell activation and is dependent on CD4 engagement. Phosphorylation on the cytoplasmic tail triggers the activation of downstream signalling molecules. TCR signalling induces production of cytokines responsible for cell survival, proliferation, and transcriptional activation. Solid arrows indicate a direct interaction. Broken arrows indicate that intermediate steps are involved.

1.7 Rationale, Hypothesis, and Objectives:

Statement of Importance: The establishment of replication-competent provirus in resting CD4+ T lymphocytes represents a significant barrier to HIV-1 curative research. The viral reservoir is highly stable, and infected cells are phenotypically indistinguishable from uninfected CD4+ T cells – thus making it difficult to selectively target these cells for eradication. Therapeutic interventions designed to eliminate this cellular reservoir have largely failed, emphasizing the need for more innovative and bold strategies to destroy this cellular reservoir. Evidence suggests that HIV preferentially infects and establishes latency within HIV-specific CD4+ T cells and that HIV latency reversal can be achieved using HIV derived proteins. Therefore, a therapeutic vaccine that represents the entire proteome of HIV within a given individual, immediately prior to antiretroviral therapy, might activate the entire cellular reservoir and initiate proviral gene transcription.

Since the discovery of the latent reservoir in the late 1990s, many strategies have been employed in attempt to reduce or eradicate these pools of latent provirus. Though many of these regimens remain unsuccessful, one strategy, aptly called the 'shock and kill' strategy for HIV eradication, is being critically investigated. Our activation vector, ACT-VEC, is a novel therapeutic vaccine strategy designed to reactivate, or 'shock', latently infected cells back into transcriptional activity. Preliminary studies reveal that ACT-VEC can potently reactivate HIV-specific CD4+ T cells, and outperforms many single and dual-drug regimens currently under clinical investigation.

Statement of Hypothesis: HIV latency reversal may be maximally achieved by using a highly diverse, heterologous formulation representing a host of patient-derived viral antigens to activate the HIV-specific memory CD4+ T cell reservoir.

Experimental Aims:

<u>**Aim 1**: Develop a heterogenous virus-like particle (VLP) vaccine for HIV-1 latency reversal</u>

ACT-VEC is a heterologous virus-like particle (VLP) formulation that encompasses the quasi-species of five chronically infected individuals.[157] We aim to utilize a yeast recombination system to ensure maximum diversity within each of the five individual donor VLPs which, in

combination, form the basis of ACT-VEC. As part of development, our ACT-VEC formulation requires genomic modifications rendering it non-infectious, including i) removal of the 5' LTR of HIV-1, ii) abrogation of the packaging signal via modification of stem loop 1 within Gag (dS.1 mutation), and iii) removal of viral RNA from the particles. Herein, we aim to perform preliminary investigations regarding phenotypic characteristics, safety, and diversity of the formulation following successful development.

Aim 2: Evaluation of ACT-VEC VLP as a latency reversing agent in donors infected and treated during the acute stage of infection

Following development, we seek to investigate the impact of a highly heterogenous virus-like particle formulation in the context of acute-stage infection. During this stage, latent reservoirs are more likely restricted to HIV-specific CD4$^+$ T cells, providing an optimal model for a targeted latency reversal investigation. Using an *in vitro* MDDC-T cell co-culture assay, the immunogenicity and latency reversing potential of our ACT-VEC vaccine candidate is compared to the most relevant LRAs currently under clinical investigation. Genomic readouts, including replication-competent induced virus, are determined via deep gene sequencing. Cellular readouts are determined using IFN-γ ELISpot and culture supernatant qRT-PCR. Furthermore, this study aims to identify potential mechanisms contributing to transcriptional reactivation of latent provirus.

Aim 3: Perform latency reversal on chronically infected, heterologous donor samples

Following the findings present within Aim 2, it is important to evaluate the effect of our VLP candidate in the context of individuals infected and diagnosed at the chronic stage of infection. As most HIV-infected individuals are diagnosed at this stage, this model is the most biologically relevant in regard to determining vaccine efficacy. Moreover, many potential complications arise that are unique to this stage of infection, including, i) the potential of T cell exhaustion/anergy, ii) higher diversity in the viral quasi-species, and iii) the establishment of alternative reservoirs due to co-infection. Taking these complications into account, it will be interesting to compare the efficacy of ACT-VEC during the acute and chronic stages of infection.

Similar to Aim 2, all latency reversal assays will be performed *in vitro* using PBMCs from consenting individuals within this respective cohort.

1.8 References

1.   CDC. Pneumocystis Pneumonia in Los Angeles. *MMWR Wkly. Rep.* (1981).

2.   Walzer, P. D., Perl, D. P. & Krogstad, D. J. Pneumocystis carinii pneumonia in the United States: Epidemiologic, diagnostic, and clinical features. *National Cancer Institute Monograph* (1976).

3.   Immunodeficiency among female sexual partners of males with acquired immune deficiency syndrome (AIDS) - New York. *MMWR. Morb. Mortal. Wkly. Rep.* (1983).

4.   Prevention of acquired immune deficiency syndrome (AIDS): report of inter-agency recommendations. *MMWR. Morb. Mortal. Wkly. Rep.* **32**, 101–103 (1983).

5.   Barre-Sinoussi, F. *et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**, 868–871 (1983).

6.   Gallo, R. C. *et al.* Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science (80-.).* (1984). doi:10.1126/science.6200936

7.   Marx, J. L. AIDS virus has new name - Perhaps. *Science* (1986). doi:10.1126/science.3008336

8.   Fran??oise Barr??-Sinoussi and Luc Montagnier share the 2008 Nobel Prize for Physiology and Medicine for their discovery of the human immunodeficiency virus (HIV). *AIDS* (2009).

9.   Zhu, T. *et al.* An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. *Nature* **391**, 594–7 (1998).

10.   Vidal, N. *et al.* Unprecedented Degree of Human Immunodeficiency Virus Type 1 (HIV-1) Group M Genetic Diversity in the Democratic Republic of Congo Suggests that the HIV-1 Pandemic Originated in Central Africa. *J. Virol.* **74**, 10498–10507 (2000).

11.   Sharp, P. M. & Hahn, B. H. Origins of HIV and the AIDS pandemic. *Cold Spring Harb. Perspect. Med.* (2011). doi:10.1101/cshperspect.a006841

12.   Sharp, P. M. & Hahn, B. H. Origins of HIV and the AIDS pandemic. *Cold Spring Harb. Perspect. Med.* **1**, (2011).

13.   Bailes, E. *et al.* Hybrid origin of SIV in chimpanzees. *Science (80-.).* (2003). doi:10.1126/science.1080657

14.   Gao, F. *et al.* Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* (1999). doi:10.1038/17130

15. UNAIDS. Fact Sheet - Global AIDS Update 2019. *UNAIDS* (2019).

16. UNAIDS. Fact Sheet 2016. *2030 | E n d i n g t h e A I D S e p i d e m i c* (2016).

17. Lu, K., Heng, X. & Summers, M. F. Structural determinants and mechanism of HIV-1 genome packaging. *Journal of Molecular Biology* (2011). doi:10.1016/j.jmb.2011.04.029

18. Li, G. & De Clercq, E. HIV Genome-Wide Protein Associations: a Review of 30 Years of Research. *Microbiol. Mol. Biol. Rev.* (2016). doi:10.1128/mmbr.00065-15

19. Baldwin, D. N. & Linial, M. L. The roles of Pol and Env in the assembly pathway of human foamy virus. *J. Virol.* (1998).

20. Freed, E. O. HIV-1 Gag proteins: Diverse functions in the virus life cycle. *Virology* (1998). doi:10.1006/viro.1998.9398

21. Fassati, A. Multiple roles of the capsid protein in the early steps of HIV-1 infection. *Virus Research* (2012). doi:10.1016/j.virusres.2012.09.012

22. Los Alamos National Laboratory. Landmarks of the HIV genome. *Human Retroviruses and AIDS* (1998).

23. Meek, T. D. *et al.* Inhibition of HIV-1 protease in infected T-lymphocytes by synthetic peptide analogues. *Nature* (1990). doi:10.1038/343090a0

24. Chan, D. C. & Kim, P. S. HIV entry and its inhibition. *Cell* (1998). doi:10.1016/S0092-8674(00)81430-0

25. Stoltzfus, C. & Madsen, J. Role of Viral Splicing Elements and Cellular RNA Binding Proteins in Regulation of HIV-1 Alternative RNA Splicing. *Curr. HIV Res.* (2005). doi:10.2174/1570162206775197655

26. Ocwieja, K. E. *et al.* Dynamic regulation of HIV-1 mRNA populations analyzed by single-molecule enrichment and long-read sequencing. *Nucleic Acids Res.* (2012). doi:10.1093/nar/gks753

27. Purcell, D. F. & Martin, M. A. Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. *J. Virol.* (1993).

28. Lützelberger, M., Reinert, L. S., Das, A. T., Berkhout, B. & Kjems, J. A novel splice donor site in the gag-pol gene is required for HIV-1 RNA stability. *J. Biol. Chem.* (2006). doi:10.1074/jbc.M513698200

29. Tazi, J. *et al.* Alternative splicing: Regulation of HIV-1 multiplication as a target for therapeutic action: Minireview. *FEBS Journal* (2010). doi:10.1111/j.1742-4658.2009.07522.x

30. Mbonye, U. & Karn, J. Transcriptional control of HIV latency: Cellular signaling pathways, epigenetics, happenstance and the hope for a cure. *Virology* **454–455**, 328–339 (2014).

31. Rice, A. P. The HIV-1 Tat Protein: Mechanism of Action and Target for HIV-1 Cure

Strategies. *Curr. Pharm. Des.* (2017). doi:10.2174/1381612823666170704130635

32. Chang, D. D. & Sharp, P. A. Regulation by HIV Rev depends upon recognition of splice sites. *Cell* (1989). doi:10.1016/0092-8674(89)90602-8

33. Emerman, M., Vazeux, R. & Peden, K. The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. *Cell* (1989). doi:10.1016/0092-8674(89)90053-6

34. Felber, B. K., Hadzopoulou-Cladaras, M., Cladaras, C., Copeland, T. & Pavlakis, G. N. rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *Proc. Natl. Acad. Sci. U. S. A.* (1989). doi:10.1073/pnas.86.5.1495

35. Malim, M. H. & Emerman, M. HIV-1 Accessory Proteins-Ensuring Viral Survival in a Hostile Environment. *Cell Host and Microbe* (2008). doi:10.1016/j.chom.2008.04.008

36. Deacon, N. J. *et al.* Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science (80-.).* (1995). doi:10.1126/science.270.5238.988

37. Roeth, J. F. & Collins, K. L. Human Immunodeficiency Virus Type 1 Nef: Adapting to Intracellular Trafficking Pathways. *Microbiol. Mol. Biol. Rev.* (2006). doi:10.1128/mmbr.00042-05

38. Chaudhuri, R., Lindwasser, O. W., Smith, W. J., Hurley, J. H. & Bonifacino, J. S. Downregulation of CD4 by Human Immunodeficiency Virus Type 1 Nef Is Dependent on Clathrin and Involves Direct Interaction of Nef with the AP2 Clathrin Adaptor. *J. Virol.* (2007). doi:10.1128/jvi.02725-06

39. Perez-Caballero, D. *et al.* Tetherin Inhibits HIV-1 Release by Directly Tethering Virions to Cells. *Cell* (2009). doi:10.1016/j.cell.2009.08.039

40. Letko, M., Booiman, T., Kootstra, N., Simon, V. & Ooms, M. Identification of the HIV-1 Vif and Human APOBEC3G Protein Interface. *Cell Rep.* (2015). doi:10.1016/j.celrep.2015.10.068

41. Le Rouzic, E. *et al.* HIV1 Vpr arrests the cell cycle by recruiting DCAF1/VprBP, a receptor of the Cul4-DDB1 ubiquitin ligase. *Cell Cycle* (2007). doi:10.4161/cc.6.2.3732

42. Belzile, J. P. *et al.* HIV-1 Vpr-mediated G2 arrest involves the DDB1-CUL4AVPRBP E3 ubiquitin ligase. *PLoS Pathog.* (2007). doi:10.1371/journal.ppat.0030085

43. Kirchhoff, F. HIV Life Cycle: Overview. in *Encyclopedia of AIDS* (2013). doi:10.1007/978-1-4614-9610-6_60-1

44. Cuevas, J. M., Geller, R., Garijo, R., López-Aldeguer, J. & Sanjuán, R. Extremely High Mutation Rate of HIV-1 In Vivo. *PLoS Biol.* (2015). doi:10.1371/journal.pbio.1002251

45. Arhel, N. Revisiting HIV-1 uncoating. *Retrovirology* (2010). doi:10.1186/1742-4690-7-96

46. De Witte, L. *et al.* Langerin is a natural barrier to HIV-1 transmission by Langerhans cells.

Nat. Med. **13**, 367–371 (2007).

47. Donahue, D. A. & Wainberg, M. A. Cellular and molecular mechanisms involved in the establishment of HIV-1 latency. *Retrovirology* (2013). doi:10.1186/1742-4690-10-11

48. Murphy, K. M. & Stockinger, B. Effector T cell plasticity: Flexibility in the face of changing circumstances. *Nature Immunology* (2010). doi:10.1038/ni.1899

49. Wei, X. *et al.* Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**, 117–22 (1995).

50. Ho, D. D. *et al.* Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**, 123–126 (1995).

51. Vatakis, D. N., Nixon, C. C. & Zack, J. A. Quiescent T cells and HIV: An unresolved relationship. *Immunologic Research* (2010). doi:10.1007/s12026-010-8171-0

52. Deng, K. *et al.* Broad CTL response is required to clear latent HIV-1 due to dominance of escape mutations. *Nature* (2015). doi:10.1038/nature14053

53. Bleul, C. C., Wu, L., Hoxie, J. A., Springer, T. A. & Mackay, C. R. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* (1997). doi:10.1073/pnas.94.5.1925

54. Zack, J. A. *et al.* HIV-1 entry into quiescent primary lymphocytes: Molecular analysis reveals a labile, latent viral structure. *Cell* (1990). doi:10.1016/0092-8674(90)90802-L

55. Stevenson, M., Stanwick, T. L., Dempsey, M. P. & Lamonica, C. A. HIV-1 replication is controlled at the level of T cell activation and proviral integration. *EMBO J.* (1990). doi:10.1002/j.1460-2075.1990.tb08274.x

56. Soriano-Sarabia, N. *et al.* Peripheral Vγ9Vδ2 T Cells Are a Novel Reservoir of Latent HIV Infection. *PLoS Pathog.* (2015). doi:10.1371/journal.ppat.1005201

57. Wightman, F. *et al.* Both CD31 + and CD31 − Naive CD4 + T Cells Are Persistent HIV Type 1–Infected Reservoirs in Individuals Receiving Antiretroviral Therapy . *J. Infect. Dis.* (2010). doi:10.1086/656721

58. Clayton, K. L., Garcia, V., Clements, J. E. & Walker, B. D. HIV Infection of Macrophages: Implications for Pathogenesis and Cure. *Pathog. Immun.* (2017). doi:10.20411/pai.v2i2.204

59. Das, A. T., Harwig, A. & Berkhout, B. The HIV-1 Tat Protein Has a Versatile Role in Activating Viral Transcription. *J. Virol.* (2011). doi:10.1128/jvi.00650-11

60. Yedavalli, V. S. R. K., Benkirane, M. & Jeang, K. T. Tat and trans-activation-responsive (TAR) RNA-independent induction of HIV-1 long terminal repeat by human and murine cyclin T1 requires Sp1. *J. Biol. Chem.* (2003). doi:10.1074/jbc.M209162200

61. Razooky, B. S., Pai, A., Aull, K., Rouzine, I. M. & Weinberger, L. S. A hardwired HIV latency program. *Cell* (2015). doi:10.1016/j.cell.2015.02.009

62.   Lassen, K. G., Ramyar, K. X., Bailey, J. R., Zhou, Y. & Siliciano, R. F. Nuclear retention of multiply spliced HIV-1 RNA in resting CD4+ T cells. *PLoS Pathog.* (2006). doi:10.1371/journal.ppat.0020068

63.   Li, J. *et al.* Long noncoding RNA NRON contributes to HIV-1 latency by specifically inducing tat protein degradation. *Nat. Commun.* (2016). doi:10.1038/ncomms11730

64.   Donahue, D. A., Kuhl, B. D., Sloan, R. D. & Wainberg, M. A. The Viral Protein Tat Can Inhibit the Establishment of HIV-1 Latency. *J. Virol.* (2012). doi:10.1128/jvi.06648-11

65.   Hütter, G. *et al.* Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N. Engl. J. Med.* **360**, 692–8 (2009).

66.   Allers, K. *et al.* Evidence for the cure of HIV infection by CCR5Delta32/Delta32 stem cell transplantation. *Blood* **117**, 2791–2799 (2011).

67.   Gupta, R. K. *et al.* HIV-1 remission following CCR5Δ32/Δ32 haematopoietic stem-cell transplantation. *Nature* (2019). doi:10.1038/s41586-019-1027-4

68.   Hütter, G. More on shift of HIV tropism in stem-cell transplantation with CCR5 delta32/Delta32 mutation. *New England Journal of Medicine* (2014). doi:10.1056/NEJMc1412279

69.   Tebas, P. *et al.* Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N. Engl. J. Med.* (2014). doi:10.1056/NEJMoa1300662

70.   Kordelas Lambros,   Jens Verheyen,   Stefan Esser. Shift of HIV Tropism in Stem-Cell Transplantation with CCR5 Delta32 Mutation. *N. Engl. J. Med.* (2014). doi:10.1056/nejmc1407921

71.   Yu, S. *et al.* Simultaneous Knockout of CXCR4 and CCR5 Genes in CD4+ T Cells via CRISPR/Cas9 Confers Resistance to Both X4- and R5-Tropic Human Immunodeficiency Virus Type 1 Infection. *Hum. Gene Ther.* (2018). doi:10.1089/hum.2017.032

72.   Liu, Z. *et al.* Genome editing of the HIV co-receptors CCR5 and CXCR4 by CRISPR-Cas9 protects CD4+ T cells from HIV-1 infection. *Cell Biosci.* (2017). doi:10.1186/s13578-017-0174-2

73.   Burke, B. P. *et al.* CCR5 as a natural and modulated target for inhibition of HIV. *Viruses* (2013). doi:10.3390/v6010054

74.   Henrich, T. J. *et al.* Long-term reduction in peripheral blood HIV type 1 reservoirs following reduced-intensity conditioning allogeneic stem cell transplantation. *J. Infect. Dis.* **207**, 1694–702 (2013).

75.   Henrich, T. J. *et al.* Antiretroviral-free HIV-1 remission and viral rebound after allogeneic stem cell transplantation: Report of 2 cases. *Ann. Intern. Med.* (2014). doi:10.7326/M14-1027

76.   Bai, Y. *et al.* Covalent fusion inhibitors targeting HIV-1 gp41 deep pocket. *Amino Acids*

(2013). doi:10.1007/s00726-012-1394-8

77. Shirakawa, K., Chavez, L., Hakre, S., Calvanese, V. & Verdin, E. Reactivation of latent HIV by histone deacetylase inhibitors. *Trends in Microbiology* (2013). doi:10.1016/j.tim.2013.02.005

78. Lehrman, G. *et al.* Depletion of latent HIV-1 infection in vivo: A proof-of-concept study. *Lancet* **366**, 549–555 (2005).

79. Liu, R. *et al.* Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* (1996). doi:10.1016/S0092-8674(00)80110-5

80. Samson, M. *et al.* Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* (1996). doi:10.1038/382722a0

81. Dragic, T. *et al.* A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *Proc. Natl. Acad. Sci. U. S. A.* (2000). doi:10.1073/pnas.090576697

82. Westby, M. *et al.* Emergence of CXCR4-Using Human Immunodeficiency Virus Type 1 (HIV-1) Variants in a Minority of HIV-1-Infected Patients following Treatment with the CCR5 Antagonist Maraviroc Is from a Pretreatment CXCR4-Using Virus Reservoir. *J. Virol.* (2006). doi:10.1128/jvi.80.10.4909-4920.2006

83. Ratcliff, A. N., Shi, W. & Arts, E. J. HIV-1 Resistance to Maraviroc Conferred by a CD4 Binding Site Mutation in the Envelope Glycoprotein gp120. *J. Virol.* (2013). doi:10.1128/jvi.01863-12

84. Arts, E. J. & Hazuda, D. J. HIV-1 antiretroviral drug therapy. *Cold Spring Harb. Perspect. Med.* (2012). doi:10.1101/cshperspect.a007161

85. Sluis-Cremer, N., Arion, D. & Parniak, M. A. Molecular mechanisms of HIV-1 resistance to nucleoside reverse transcriptase inhibitors (NRTIs). *Cellular and Molecular Life Sciences* (2000). doi:10.1007/PL00000626

86. Günthard, H. F. *et al.* Antiretroviral Drugs for Treatment and Prevention of HIV Infection in Adults. *JAMA* (2016). doi:10.1001/jama.2016.8900

87. Shen, L. *et al.* Dose-response curve slope sets class-specific limits on inhibitory potential of anti-HIV drugs. *Nat. Med.* (2008). doi:10.1038/nm1777

88. Sluis-Cremer, N., Temiz, N. & Bahar, I. Conformational Changes in HIV-1 Reverse Transcriptase Induced by Nonnucleoside Reverse Transcriptase Inhibitor Binding. *Curr. HIV Res.* (2005). doi:10.2174/1570162043351093

89. Soriano, V. & de Mendoza, C. Genetic mechanisms of resistance to NRTI and NNRTI. *HIV Clinical Trials* (2002). doi:10.1310/06DD-UN4D-9AW2-RLBY

90. Craigie, R. HIV Integrase, a Brief Overview from Chemistry to Therapeutics. *Journal of*

Biological Chemistry (2001). doi:10.1074/jbc.R100027200

91.   Hazuda, D. J. *et al.* Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. *Science (80-.).* (2000). doi:10.1126/science.287.5453.646

92.   Anthony, N. HIV-1 Integrase: A Target for New AIDS Chemotherapeutics. *Curr. Top. Med. Chem.* (2005). doi:10.2174/1568026043388448

93.   Walmsley, S. *et al.* Dolutegravir (DTG; S/GSK1349572) + Abacavir/Lamivudine Once Daily Statistically Superior to Tenofovir/Emtricitabine/Efavirenz: 48-Week Results - SINGLE (ING114467). *52nd ICAAC-Sept. 9-12-San Francisco* (2012).

94.   Flexner, C. HIV-protease inhibitors. *New England Journal of Medicine* (1998). doi:10.1056/NEJM199804303381808

95.   Zaidi, N. *et al.* A novel cell penetrating aspartic protease inhibitor blocks processing and presentation of tetanus toxoid more efficiently than pepstatin A. *Biochem. Biophys. Res. Commun.* (2007). doi:10.1016/j.bbrc.2007.09.114

96.   King, J. R., Wynn, H., Brundage, R. & Acosta, E. P. Pharmacokinetic enhancement of protease inhibitor therapy. *Clinical Pharmacokinetics* (2004). doi:10.2165/00003088-200443050-00003

97.   UNAIDS. *Global HIV & AIDS statistics — 2019 fact sheet | UNAIDS*. (2019).

98.   Chesney, M. A. Factors Affecting Adherence to Antiretroviral Therapy. *Clin. Infect. Dis.* (2000). doi:10.1086/313849

99.   Deeks, E. D. & Perry, C. M. Efavirenz/Emtricitabine/Tenofovir Disoproxil Fumarate Single-Tablet Regimen (Atripla®). *Drugs* (2010). doi:10.2165/11203800-000000000-00000

100.   Girouard, M. P. *et al.* The Cost-effectiveness and Budget Impact of 2-Drug Dolutegravir-Lamivudine Regimens for the Treatment of HIV Infection in the United States. *Clin. Infect. Dis.* (2015). doi:10.1093/cid/civ981

101.   Samji, H. *et al.* Closing the gap: Increases in life expectancy among treated HIV-positive individuals in the United States and Canada. *PLoS One* (2013). doi:10.1371/journal.pone.0081355

102.   Lewden, C. *et al.* All-cause mortality in treated HIV-infected adults with CD4 ≥500/mm3 compared with the general population: Evidence from a large European observational cohort collaboration. *Int. J. Epidemiol.* (2012). doi:10.1093/ije/dyr164

103.   Freiberg, M. S. *et al.* HIV infection and the risk of acute myocardial infarction. *JAMA Intern. Med.* (2013). doi:10.1001/jamainternmed.2013.3728

104.   Brown, T. T. & Qaqish, R. B. Antiretroviral therapy and the prevalence of osteopenia and osteoporosis: A meta-analytic review. *AIDS* (2006). doi:10.1097/QAD.0b013e32801022eb

105.   Brown, T. T. *et al.* Antiretroviral therapy and the prevalence and incidence of diabetes mellitus in the multicenter AIDS cohort study. *Arch. Intern. Med.* (2005).

doi:10.1001/archinte.165.10.1179

106.   Hileman, C. O. & Funderburg, N. T. Inflammation, Immune Activation, and Antiretroviral Therapy in HIV. *Current HIV/AIDS Reports* (2017). doi:10.1007/s11904-017-0356-x

107.   Bahrami, H. *et al.* Inflammatory markers associated with subclinical coronary artery disease: The multicenter AIDS cohort study. *J. Am. Heart Assoc.* (2016). doi:10.1161/JAHA.116.003371

108.   Chun, T. *et al.* Persistence of HIV in Gut-Associated Lymphoid Tissue despite Long-Term Antiretroviral Therapy. *J. Infect. Dis.* (2008). doi:10.1086/527324

109.   Chun, T. W. *et al.* HIV-infected individuals receiving effective antiviral therapy for extended periods of time continually replenish their viral reservoir. *J. Clin. Invest.* (2005). doi:10.1172/JCI26197

110.   Crane, M. *et al.* Lipopolysaccharide, immune activation, and liver abnormalities in HIV/hepatitis B virus (HBV)-coinfected individuals receiving HBV-active combination antiretroviral therapy. *J. Infect. Dis.* (2014). doi:10.1093/infdis/jiu119

111.   Lederman, M. M., Funderburg, N. T., Sekaly, R. P., Klatt, N. R. & Hunt, P. W. Residual immune dysregulation syndrome in treated HIV infection. in *Advances in Immunology* (2013). doi:10.1016/B978-0-12-407707-2.00002-3

112.   Sáez-Cirión, A. *et al.* Post-Treatment HIV-1 Controllers with a Long-Term Virological Remission after the Interruption of Early Initiated Antiretroviral Therapy ANRS VISCONTI Study. *PLoS Pathog.* **9**, (2013).

113.   Persaud, D. *et al.* Absence of Detectable HIV-1 Viremia after Treatment Cessation in an Infant. *N. Engl. J. Med.* **369**, 1828–1835 (2013).

114.   Luzuriaga, K. *et al.* Viremic relapse after HIV-1 remission in a perinatally infected child. *New England Journal of Medicine* (2014). doi:10.1056/NEJMc1413931

115.   Whitney, J. B. *et al.* Rapid seeding of the viral reservoir prior to SIV viraemia in rhesus monkeys. *Nature* **512**, 74–77 (2014).

116.   Eriksson, S. *et al.* Comparative Analysis of Measures of Viral Reservoirs in HIV-1 Eradication Studies. *PLoS Pathog.* (2013). doi:10.1371/journal.ppat.1003174

117.   Strain, M. C. *et al.* Effect of Treatment, during Primary Infection, on Establishment and Clearance of Cellular Reservoirs of HIV-1. *J. Infect. Dis.* (2005). doi:10.1086/428777

118.   Abrahams, M. R. *et al.* The replication-competent HIV-1 latent reservoir is primarily established near the time of therapy initiation. *Sci. Transl. Med.* (2019). doi:10.1126/scitranslmed.aaw5589

119.   Mbonye, U. & Karn, J. The Molecular Basis for Human Immunodeficiency Virus Latency. *Annu. Rev. Virol.* (2017). doi:10.1146/annurev-virology-101416-041646

120.   Kim, Y., Anderson, J. L. & Lewin, S. R. Getting the "Kill" into "Shock and Kill": Strategies to

Eliminate Latent HIV. *Cell Host and Microbe* (2018). doi:10.1016/j.chom.2017.12.004

121.   Siliciano, J. D. *et al.* Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. *Nat. Med.* **9**, 727–728 (2003).

122.   Williams, S. A. *et al.* Prostratin antagonizes HIV latency by activating NF-κB. *J. Biol. Chem.* **279**, 42008–42017 (2004).

123.   Keedy, K. S. *et al.* A Limited Group of Class I Histone Deacetylases Acts To Repress Human Immunodeficiency Virus Type 1 Expression. *J. Virol.* (2009). doi:10.1128/jvi.02585-08

124.   Jiang, G., Espeseth, A., Hazuda, D. J. & Margolis, D. M. c-Myc and Sp1 Contribute to Proviral Latency by Recruiting Histone Deacetylase 1 to the Human Immunodeficiency Virus Type 1 Promoter. *J. Virol.* (2007). doi:10.1128/jvi.01208-07

125.   Verdin, E., Paras, P. & Van Lint, C. Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. *EMBO J.* (1993). doi:10.1002/j.1460-2075.1993.tb05994.x

126.   De Ruijter, A. J. M., Van Gennip, A. H., Caron, H. N., Kemp, S. & Van Kuilenburg, A. B. P. Histone deacetylases (HDACs): Characterization of the classical HDAC family. *Biochemical Journal* (2003). doi:10.1042/BJ20021321

127.   Martínez-Iglesias, O. *et al.* Histone deacetylase inhibitors: Mechanism of action and therapeutic use in cancer. *Clin. Transl. Oncol.* (2008). doi:10.1007/s12094-008-0221-x

128.   Williams, S. A. *et al.* NF-κB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. *EMBO J.* **25**, 139–149 (2006).

129.   Contreras, X. *et al.* Suberoylanilide hydroxamic acid reactivates HIV from latently infected cells. *J. Biol. Chem.* **284**, 6782–6789 (2009).

130.   Siliciano, J. D. *et al.* Stability of the Latent Reservoir for HIV-1 in Patients Receiving Valproic Acid. *J. Infect. Dis.* **195**, 833–836 (2007).

131.   Mann, B. S., Johnson, J. R., Cohen, M. H., Justice, R. & Pazdur, R. FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma. *Oncologist* **12**, 1247–52 (2007).

132.   Archin, N. M. *et al.* Expression of latent human immunodeficiency type 1 is induced by novel and selective histone deacetylase inhibitors. *AIDS* **23**, 1799–806 (2009).

133.   Archin, N. M. *et al.* Expression of Latent HIV Induced by the Potent HDAC Inhibitor Suberoylanilide Hydroxamic Acid. *AIDS Res. Hum. Retroviruses* **25**, 207–212 (2009).

134.   Archin, N. M. *et al.* Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. *Nature* **487**, 482–485 (2012).

135.   Archin, N. M. *et al.* HIV-1 Expression Within Resting CD4+ T Cells After Multiple Doses of Vorinostat. *J. Infect. Dis.* **210**, 728–735 (2014).

136. Lucera, M. B. *et al.* The Histone Deacetylase Inhibitor Vorinostat (SAHA) Increases the Susceptibility of Uninfected CD4+ T Cells to HIV by Increasing the Kinetics and Efficiency of Postentry Viral Events. *J. Virol.* **88**, 10803–10812 (2014).

137. Rasmussen, T. A. *et al.* Panobinostat, a histone deacetylase inhibitor, for latent-virus reactivation in HIV-infected patients on suppressive antiretroviral therapy: a phase 1/2, single group, clinical trial. *Lancet HIV* **1**, e13-21 (2014).

138. Wei, D. G. *et al.* Histone Deacetylase Inhibitor Romidepsin Induces HIV Expression in CD4 T Cells from Patients on Suppressive Antiretroviral Therapy at Concentrations Achieved by Clinical Dosing. *PLoS Pathog.* **10**, (2014).

139. Rasmussen, T. A. *et al.* Comparison of HDAC inhibitors in clinical development: effect on HIV production in latently infected cells and T-cell activation. *Hum. Vaccin. Immunother.* **9**, 993–1001 (2013).

140. Søgaard, O. S. *et al.* The Depsipeptide Romidepsin Reverses HIV-1 Latency In Vivo. *PLoS Pathog.* **11**, (2015).

141. Shan, L. *et al.* Stimulation of HIV-1-Specific Cytolytic T Lymphocytes Facilitates Elimination of Latent Viral Reservoir after Virus Reactivation. *Immunity* (2012). doi:10.1016/j.immuni.2012.01.014

142. Jones, R. B. *et al.* A Subset of Latency-Reversing Agents Expose HIV-Infected Resting CD4 + T-Cells to Recognition by Cytotoxic T-Lymphocytes. *PLoS Pathog.* (2016). doi:10.1371/journal.ppat.1005545

143. Pace, M. *et al.* Histone Deacetylase Inhibitors Enhance CD4 T Cell Susceptibility to NK Cell Killing but Reduce NK Cell Function. *PLoS Pathog.* (2016). doi:10.1371/journal.ppat.1005782

144. Chew, G. M. *et al.* TIGIT Marks Exhausted T Cells, Correlates with Disease Progression, and Serves as a Target for Immune Restoration in HIV and SIV Infection. *PLoS Pathog.* (2016). doi:10.1371/journal.ppat.1005349

145. Li, Z., Guo, J., Wu, Y. & Zhou, Q. The BET bromodomain inhibitor JQ1 activates HIV latency through antagonizing Brd4 inhibition of Tat-transactivation. *Nucleic Acids Res.* **41**, 277–287 (2013).

146. Darcis, G. *et al.* An In-Depth Comparison of Latency-Reversing Agent Combinations in Various In Vitro and Ex Vivo HIV-1 Latency Models Identified Bryostatin-1+JQ1 and Ingenol-B+JQ1 to Potently Reactivate Viral Gene Expression. *PLoS Pathog.* **11**, (2015).

147. Khoury, G. *et al.* HIV latency reversing agents act through Tat post translational modifications. *Retrovirology* (2018). doi:10.1186/s12977-018-0421-6

148. Banerjee, C. *et al.* BET bromodomain inhibition as a novel strategy for reactivation of HIV-1. *J. Leukoc. Biol.* **92**, 1147–1154 (2012).

149. Laird, G. M. *et al.* Ex vivo analysis identifies effective HIV-1 latency-reversing drug

combinations. *J. Clin. Invest.* **125**, 1901–1912 (2015).

150. Ramsdell, J. S., Pettit, G. R. & Tashjian, A. H. Three activators of protein kinase C, bryostatins, dioleins, and phorbol esters, show differing specificities of action on GH4 pituitary cells. *J. Biol. Chem.* **261**, 17073–17080 (1986).

151. Bullen, C. K., Laird, G. M., Durand, C. M., Siliciano, J. D. & Siliciano, R. F. New ex vivo approaches distinguish effective and ineffective single agents for reversing HIV-1 latency in vivo. *Nat. Med.* **20**, 425–429 (2014).

152. Gutiérrez, C. *et al.* Bryostatin-1 for latent virus reactivation in HIV-infected patients on antiretroviral therapy. *AIDS* **30**, 1385–92 (2016).

153. Kulkosky, J. *et al.* Prostratin: Activation of latent HIV-1 expression suggests a potential inductive adjuvant therapy for HAART. *Blood* **98**, 3006–3015 (2001).

154. Beans, E. J. *et al.* Highly potent, synthetically accessible prostratin analogs induce latent HIV expression in vitro and ex vivo. *Proc. Natl. Acad. Sci.* **110**, 11698–11703 (2013).

155. Dechristopher, B. A. *et al.* Designed, synthetically accessible bryostatin analogues potently induce activation of latent HIV reservoirs in vitro. *Nat. Chem.* **4**, 705–710 (2012).

156. Chroboczek, J., Szurgot, I. & Szolajska, E. Virus-like particles as vaccine. *Acta Biochimica Polonica* (2014). doi:10.18388/abp.2014_1875

157. Pankrac, J. *et al.* A heterogeneous human immunodeficiency virus-like particle (VLP) formulation produced by a novel vector system. *npj Vaccines* **3**, 2 (2018).

158. Bachmann, M. F. & Jennings, G. T. Vaccine delivery: A matter of size, geometry, kinetics and molecular patterns. *Nature Reviews Immunology* (2010). doi:10.1038/nri2868

159. Lingappa, J. R., Reed, J. C., Tanaka, M., Chutiraka, K. & Robinson, B. A. How HIV-1 Gag assembles in cells: Putting together pieces of the puzzle. *Virus Res.* (2014). doi:10.1016/j.virusres.2014.07.001

160. Pankrac, J. *et al.* A heterogeneous human immunodeficiency virus-like particle (VLP) formulation produced by a novel vector system. *npj Vaccines* **3**, (2018).

161. Chen, Q. & Lai, H. Plant-derived virus-like particles as vaccines. *Human Vaccines and Immunotherapeutics* (2013). doi:10.4161/hv.22218

162. Arevalo, M. T., Wong, T. M. & Ross, T. M. Expression and purification of virus-like particles for vaccination. *J. Vis. Exp.* (2016). doi:10.3791/54041

163. Dudley, D. M. *et al.* A novel yeast-based recombination method to clone and propagate diverse HIV-1 isolates. *Biotechniques* (2009). doi:10.2144/000113119

164. Mohsen, M. O., Speiser, D. E., Knuth, A. & Bachmann, M. F. Virus-like particles for vaccination against cancer. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* (2019). doi:10.1002/wnan.1579

165.   Pankrac, J., Klein, K. & Mann, J. F. S. Eradication of HIV-1 latent reservoirs through therapeutic vaccination. *AIDS Res. Ther.* **14**, (2017).

166.   Bachmann, M. F. *et al.* TAP1-independent loading of class I molecules by exogenous viral proteins. *Eur. J. Immunol.* (1995). doi:10.1002/eji.1830250637

167.   Storni, T. & Bachmann, M. F. Loading of MHC Class I and II Presentation Pathways by Exogenous Antigens: A Quantitative In Vivo Comparison. *J. Immunol.* (2004). doi:10.4049/jimmunol.172.10.6129

168.   Hjelm, B. E., Kilbourne, J. & Herbst-Kralovetz, M. M. TLR7 and 9 agonists are highly effective mucosal adjuvants for norovirus virus-like particle vaccines. *Hum. Vaccines Immunother.* (2014). doi:10.4161/hv.27147

169.   Bachmann, M. F. *et al.* The influence of antigen organization on B cell responsiveness. *Science (80-.).* (1993). doi:10.1126/science.8248784

170.   Stano, A. *et al.* Dense Array of Spikes on HIV-1 Virion Particles. *J. Virol.* (2017). doi:10.1128/jvi.00415-17

171.   Jegerlehner, A. *et al.* Regulation of IgG antibody responses by epitope density and CD21-mediated costimulation. *European Journal of Immunology* (2002). doi:10.1002/1521-4141(200211)32:11<3305::AID-IMMU3305>3.0.CO;2-J

172.   Brune, K. D. *et al.* Plug-and-Display: decoration of Virus-Like Particles via isopeptide bonds for modular immunization. *Sci. Rep.* (2016). doi:10.1038/srep19234

173.   Gomes, A. C., Roesti, E. S., El-Turabi, A. & Bachmann, M. F. Type of RNA packed in VLPs impacts IgG class switching—implications for an influenza vaccine design. *Vaccines* (2019). doi:10.3390/vaccines7020047

174.   Douek, D. C. *et al.* HIV preferentially infects HIV-specific CD4+ T cells. *Nature* **417**, 95–98 (2002).

175.   Demoustier, A. *et al.* In patients on prolonged HAART, a significant pool of HIV infected CD4 T cells are HIV-specific. *AIDS* **16**, 1749–1754 (2002).

176.   Mohammadi, P. *et al.* Dynamics of HIV Latency and Reactivation in a Primary CD4+ T Cell Model. *PLoS Pathog.* (2014). doi:10.1371/journal.ppat.1004156

177.   Shete, A. *et al.* Short Communication: HIV Antigen-Specific Reactivation of HIV Infection from Cellular Reservoirs: Implications in the Settings of Therapeutic Vaccinations. *AIDS Res. Hum. Retroviruses* **28**, 835–843 (2012).

178.   Collin, M. & Bigley, V. Human dendritic cell subsets: an update. *Immunology* (2018). doi:10.1111/imm.12888

179.   Posch, W., Lass-Flörl, C. & Wilflingseder, D. Generation of human monocyte-derived dendritic cells from whole blood. *J. Vis. Exp.* (2016). doi:10.3791/54968

180.   Viney, J. L. Dendritic cell subsets: The ultimate T cell differentiation decision makers? *Gut*

(1999). doi:10.1136/gut.45.5.640

181. Liu, K. *et al.* In Vivo Analysis of Dendritic Cell Development and Homeostasis. *Science (80-.).* (2009). doi:10.1126/science.1170540

182. Cheong, C. *et al.* Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209 + dendritic cells for immune T cell areas. *Cell* (2010). doi:10.1016/j.cell.2010.09.039

183. Santambrogio, L. *et al.* Extracellular antigen processing and presentation by immature dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* (1999). doi:10.1073/pnas.96.26.15056

184. Qi, Q. *et al.* Diversity and clonal selection in the human T-cell repertoire. *Proc. Natl. Acad. Sci. U. S. A.* (2014). doi:10.1073/pnas.1409155111

185. Klein, L., Kyewski, B., Allen, P. M. & Hogquist, K. A. Positive and negative selection of the T cell repertoire: What thymocytes see (and don't see). *Nature Reviews Immunology* (2014). doi:10.1038/nri3667

186. Kumar, B. V., Connors, T. J. & Farber, D. L. Human T Cell Development, Localization, and Function throughout Life. *Immunity* (2018). doi:10.1016/j.immuni.2018.01.007

187. Baldwin, T. A., Hogquist, K. A. & Jameson, S. C. The Fourth Way? Harnessing Aggressive Tendencies in the Thymus. *J. Immunol.* (2004). doi:10.4049/jimmunol.173.11.6515

188. Haynes, B. F., Markert, M. L., Sempowski, G. D., Patel, D. D. & Hale, L. P. The Role of the Thymus in Immune Reconstitution in Aging, Bone Marrow Transplantation, and HIV-1 Infection. *Annu. Rev. Immunol.* (2000). doi:10.1146/annurev.immunol.18.1.529

189. Thome, J. J. C. *et al.* Long-term maintenance of human naïve T cells through in situ homeostasis in lymphoid tissue sites. *Sci. Immunol.* (2016). doi:10.1126/sciimmunol.aah6506

190. Gourley, T. S., Wherry, E. J., Masopust, D. & Ahmed, R. Generation and maintenance of immunological memory. *Semin. Immunol.* (2004). doi:10.1016/j.smim.2004.08.013

191. Kaech, S. M. & Wherry, E. J. Heterogeneity and Cell-Fate Decisions in Effector and Memory CD8+ T Cell Differentiation during Viral Infection. *Immunity* (2007). doi:10.1016/j.immuni.2007.08.007

192. Wieten, R. W. *et al.* A single 17D yellow fever vaccination provides lifelong immunity; characterization of yellow-fever-specific neutralizing antibody and T-cell responses after vaccination. *PLoS One* (2016). doi:10.1371/journal.pone.0149871

193. Miller, J. D. *et al.* Human Effector and Memory CD8+ T Cell Responses to Smallpox and Yellow Fever Vaccines. *Immunity* (2008). doi:10.1016/j.immuni.2008.02.020

194. Jekle, A. *et al.* In Vivo Evolution of Human Immunodeficiency Virus Type 1 toward Increased Pathogenicity through CXCR4-Mediated Killing of Uninfected CD4 T Cells. *J. Virol.* (2003). doi:10.1128/jvi.77.10.5846-5854.2003

195. Herbein, G., Van Lint, C., Lovett, J. L. & Verdin, E. Distinct mechanisms trigger apoptosis

in human immunodeficiency virus type 1-infected and in uninfected bystander T lymphocytes. *J. Virol.* (1998).

196. Finkel, T. H. *et al.* Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. *Nat. Med.* (1995). doi:10.1038/nm0295-129

197. Mitra, D. HIV-1 upregulates Fas ligand expression in CD4+ T cells in vitro and in vivo: Association with Fas-mediated apoptosis and modulation by aurintricarboxylic acid. *Immunology* (1996). doi:10.1046/j.1365-2567.1996.510589.x

198. Kottilil, S. *et al.* Innate immunity in HIV infection: Enhanced susceptibility to CD95-mediated natural killer cell death and turnover induced by HIV viremia. *J. Acquir. Immune Defic. Syndr.* (2007). doi:10.1097/QAI.0b013e3180dc9909

199. Badley, A. D. *et al.* Upregulation of Fas ligand expression by human immunodeficiency virus in human macrophages mediates apoptosis of uninfected T lymphocytes. *J. Virol.* (1996).

200. Patki, A. H., Georges, D. L. & Lederman, M. M. CD4+-T-cell counts, spontaneous apoptosis, and Fas expression in peripheral blood mononuclear cells obtained from human immunodeficiency virus type 1-infected subjects. *Clin. Diagn. Lab. Immunol.* (1997).

201. Cummins, N. W. & Badley, A. D. Mechanisms of HIV-associated lymphocyte apoptosis: 2010. *Cell Death and Disease* (2010). doi:10.1038/cddis.2010.77

202. Diaz, M. *et al.* T cells containing T cell receptor excision circles are inversely related to HIV replication and are selectively and rapidly released into circulation with antiretroviral treatment. *AIDS* (2003). doi:10.1097/00002030-200305230-00005

203. Oyaizu, N. *et al.* Cross-linking of CD4 molecules upregulates Fas antigen expression in lymphocytes by inducing interferon-?? and tumor necrosis factor-?? secretion. *Blood* (1994). doi:10.1182/blood.v84.8.2622.bloodjournal8482622

204. Lum, J. J., Schnepple, D. J. & Badley, A. D. Acquired T-cell sensitivity to TRAIL mediated killing during HIV infection is regulated by CXCR4-gp120 interactions. *AIDS* (2005). doi:10.1097/01.aids.0000176212.16205.23

205. Perfettini, J. L. *et al.* NF-κB and p53 Are the Dominant Apoptosis-inducing Transcription Factors Elicited by the HIV-1 Envelope. *J. Exp. Med.* (2004). doi:10.1084/jem.20031216

206. Vendeville, A. *et al.* HIV-1 Tat Enters T Cells Using Coated Pits before Translocating from Acidified Endosomes and Eliciting Biological Responses. *Mol. Biol. Cell* (2004). doi:10.1091/mbc.E03-12-0921

207. Yang, Y. *et al.* Monocytes Treated with Human Immunodeficiency Virus Tat Kill Uninfected CD4+ Cells by a Tumor Necrosis Factor-Related Apoptosis-Induced Ligand-Mediated Mechanism. *J. Virol.* (2003). doi:10.1128/jvi.77.12.6700-6708.2003

208. Zauli, G. *et al.* Human immunodeficiency virus type 1 Nef protein sensitizes CD4+ T lymphoid cells to apoptosis via functional upregulation of the CD95/CD95 ligand pathway. *Blood* (1999). doi:10.1182/blood.v93.3.1000.403a12_1000_1010

209. Muthumani, K. *et al.* Human Immunodeficiency Virus Type 1 Nef Induces Programmed Death 1 Expression through a p38 Mitogen-Activated Protein Kinase-Dependent Mechanism. *J. Virol.* (2008). doi:10.1128/jvi.00485-08

210. Lenassi, M. *et al.* HIV Nef is secreted in exosomes and triggers apoptosis in bystander CD4+ T cells. *Traffic* (2010). doi:10.1111/j.1600-0854.2009.01006.x

211. Ayyavoo, V. *et al.* HIV-1 Vpr suppresses immune activation and apoptosis through regulation of nuclear factor κB. *Nat. Med.* (1997). doi:10.1038/nm1097-1117

212. Jacotot, E. *et al.* The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore. *J. Exp. Med.* (2000). doi:10.1084/jem.191.1.33

213. Doitsh, G. *et al.* Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. *Nature* (2014). doi:10.1038/nature12940

214. Doitsh, G. & Greene, W. C. Dissecting How CD4 T Cells Are Lost during HIV Infection. *Cell Host and Microbe* (2016). doi:10.1016/j.chom.2016.02.012

215. Doitsh, G. *et al.* Abortive HIV infection mediates CD4 T cell depletion and inflammation in human lymphoid tissue. *Cell* (2010). doi:10.1016/j.cell.2010.11.001

216. Zhou, Y., Zhang, H., Siliciano, J. D. & Siliciano, R. F. Kinetics of Human Immunodeficiency Virus Type 1 Decay following Entry into Resting CD4+ T Cells. *J. Virol.* (2005). doi:10.1128/jvi.79.4.2199-2210.2005

217. Monroe, K. M. *et al.* IFI16 DNA sensor is required for death of lymphoid CD4 T cells abortively infected with HIV. *Science (80-.).* (2014). doi:10.1126/science.1243640

218. Courtney, A. H., Lo, W. L. & Weiss, A. TCR Signaling: Mechanisms of Initiation and Propagation. *Trends in Biochemical Sciences* (2018). doi:10.1016/j.tibs.2017.11.008

219. Balagopalan, L., Coussens, N. P., Sherman, E., Samelson, L. E. & Sommers, C. L. The LAT story: a tale of cooperativity, coordination, and choreography. *Cold Spring Harbor perspectives in biology* (2010). doi:10.1101/cshperspect.a005512

220. Zhang, W. *et al.* Association of Grb2, Gads, and phospholipase C-γ1 with phosphorylated LAT tyrosine residues: Effect of LAT tyrosine mutations on T cell antigen receptor-mediated signaling. *J. Biol. Chem.* (2000). doi:10.1074/jbc.M000404200

221. Andreotti, A. H., Schwartzberg, P. L., Joseph, R. E. & Berg, L. J. T-cell signaling regulated by the Tec family kinase, Itk. *Cold Spring Harbor perspectives in biology* (2010). doi:10.1101/cshperspect.a002287

222. Jacobs, S. R. *et al.* Glucose Uptake Is Limiting in T Cell Activation and Requires CD28-

Mediated Akt-Dependent and Independent Pathways. *J. Immunol.* (2008). doi:10.4049/jimmunol.180.7.4476

223. Frauwirth, K. A. *et al.* The CD28 signaling pathway regulates glucose metabolism. *Immunity* (2002). doi:10.1016/S1074-7613(02)00323-0

224. Coloff, J. L. *et al.* Akt requires glucose metabolism to suppress Puma expression and prevent apoptosis of leukemic T cells. *J. Biol. Chem.* (2011). doi:10.1074/jbc.M110.179101

225. Zheng, Y., Delgoffe, G. M., Meyer, C. F., Chan, W. & Powell, J. D. Anergic T Cells Are Metabolically Anergic. *J. Immunol.* (2009). doi:10.4049/jimmunol.0803510

226. Powell, J. D., Lerner, C. G. & Schwartz, R. H. Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. *J. Immunol.* (1999).

Title: <u>A heterogeneous human immunodeficiency virus-like particle (VLP) formulation produced by a novel vector system</u>

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2.1 Introduction

Since it's identification as the etiological agent behind Acquired Immunodeficiency Syndrome (AIDS), HIV-1 has become a global pandemic and major public health concern. Despite the success of antiretroviral therapy (ART) at reducing viral loads and preventing CD4$^+$ T cell loss, infection endures owing to the establishment of a transcriptionally silent latent reservoir in CD4$^+$ T cells[1]. As natural immunity is not protective against HIV-1, the development of an effective vaccine strategy remains the best countermeasure to the advancing pandemic.

Despite a lack of natural immunity against HIV-1 infection and few correlates of protection to guide vaccine development, it is anticipated anti-HIV-1 vaccines should elicit potent cellular and humoral immune responses. Apart from the modest protection observed in the RV144 trial, HIV vaccines have failed to induce any protective efficacy in clinical trials[2,3]. Alternatively, there is a renewed focus on therapeutic HIV vaccine development and the elimination of cell-associated or cell-free HIV in patients treated with ART. Thus, it is a priority to develop improved vaccine immunogens that can activate both cellular and humoral immune responses with a view to not only protect against HIV acquisition, but also to eliminate viral reservoirs such that viral remission or even a cure is achieved. A significant impediment to vaccine efficacy is thought to be the sheer HIV-1 sequence diversity[4]. A therapeutic vaccine must elicit an immune response that overcomes the high intra-patient sequence diversity and any immune-escape mutations. Polyvalent Env vaccines are one approach designed to overcome the viral diversity and involves the use of heterologous HIV-1 Env mixtures to expose developing immune responses to diverse Env conformations[5–8]. Nonetheless, a monovalent, let alone polyvalent, native Env structure is difficult to achieve outside of the HIV particle. Polyvalent Env vaccines may provide vaccine breadth, and harness the elusive neutralizing antibody response, which has both therapeutic and preventative benefits[9–11]. This does not discount that non-neutralizing antibodies may play a vital role in vaccine-mediated protection[12]. From another standpoint, eliciting broad cellular immune responses through vaccination is also highly desirable considering CTL responses can reduce viral loads, thereby contributing to the elimination of productively infected cells[13].

In pursuit of more efficacious HIV vaccines, efforts have focused on virus-like particles (VLPs), due to their capability of presenting native Env spikes on their surfaces as well as their capacity to be endocytosed and presented to T cells in the context of both MHC class I and II[14]. VLPs are defined as viral particles that have undergone protein and lipid self assembly to generate non-infectious viral particles (VPs) devoid of any viral genetic material. Furthermore, VLPs may be morphologically indistinguishable from wild type infectious virus and present the entire HIV proteome as antigen.

Herein we describe a safe, chimeric HIV-1 VLP vector system, capable of accommodating near full-length HIV genomes and captures the HIV diversity present within patient samples. The vector system was designed to generate VLPs for testing as anti-HIV therapeutic purposes[15]. Viral RNA from plasma of HIV-infected volunteers was reverse transcribed and cloned into a DNA vector for yeast-based recombination/gap repair. Full-length patient-derived HIV-1 genomes were mutated during the cloning process to stop reverse transcription, integrase (IN) activity, and genomic RNA packaging into VLPs. The VLPs express the processed HIV-1 proteome, were morphologically indistinguishable from HIV VPs, and were capable of stimulating both CD4 T cell and cytotoxic responses in heterologous patient samples. Finally, the VLPs were combined to form a highly diverse vaccine formulation called Heterologous Clade B Activating-Vector (Het_B_ACT-VEC), which shared the phenotypic and antigenic properties of the aforementioned VLPs.

2.2 Results

2.2.1 Yeast-based gap/repair recombination to clone full length HIV genomes from patients

Here we describe the ease of full genome cloning to produce patient-derived viral particles (VP, containing the viral genome) and virus-like particles (VLPs, lacking virus genome), the latter of which is currently being tested as a therapeutic vaccine (Mann; Manuscript Accepted). In addition, we will describe the representative sampling of the HIV population from plasma of infected patients into DNA vectors used to produce the vaccine constructs. Ultimately, the VP of different patients can be combined to generate heterogenous, near full length, multivalent

vaccines for both therapeutic (as part of amfAR preclinical studies) and preventative modalities (as part of the European HIV Vaccine Initiative).

A total of five HIV⁺ plasmas from infected volunteers, diagnosed at chronic stage of infection, were used to generate our vaccines. Due to the sheer genetic heterogeneity of HIV-1, chronic samples were chosen to clone viral particle preparations as they exhibit extensive diversity compared to acute samples or transmitted/founder clones, and therefore, may provide better vaccine breadth. Following reverse transcription (RT), we used external-nested PCR to amplify the full genome of HIV in two halves, overlapping by 113 bp within integrase. These overlapping 5' and 3' patient-specific HIV-1 DNA fragments were transfected into S. cerevisae along with linearized pREC-nflΔgenome/URA3. Following a double recombination event in yeast all resulting colonies were harvested to acquire maximum viral diversity – a process well described in multiple articles (Figure 2-6S)[16–18]. The DNA's were then transfected into 293T cells to produce VPs. As shown in Fig.2-1 A-C, we detected significant levels of HIV-1 capsid p24 and RT activity in the cell-free supernatants with each of the pREC-nfl plasmids containing the genomes of the patient-derived HIV (VP1, VP2, VP3, VP4 and VP5).

We also assessed and verified the presence of p24 and reverse transcriptase activity in 293T cells transfected with pREC-nfl plasmids (Figure 2-1A and B). Env expression was confirmed using a viral tropism (Veritrop) assay (Figure 2-1C). The Veritrop assay was done by transfecting 293T cells with the pREC-nfl plasmids and mixing the cells with CD4⁺/CCR5⁺ U87 cells which harbor the pDM128FLUC plasmid as previously described[19]. Here cell fusion and light emission occurs when 293T cells express functional HIV-1 gp120/gp41 Env, Rev, and Tat from the pREC-nfl vectors. Env binds CD4⁺/CCR5⁺ and mediates 293T/U87 cell fusion, permitting luciferase protein expression. All VP and VLP pREC-nfl vectors had similar levels of light emission, indicative of similar levels of Env, Rev, and Tat.
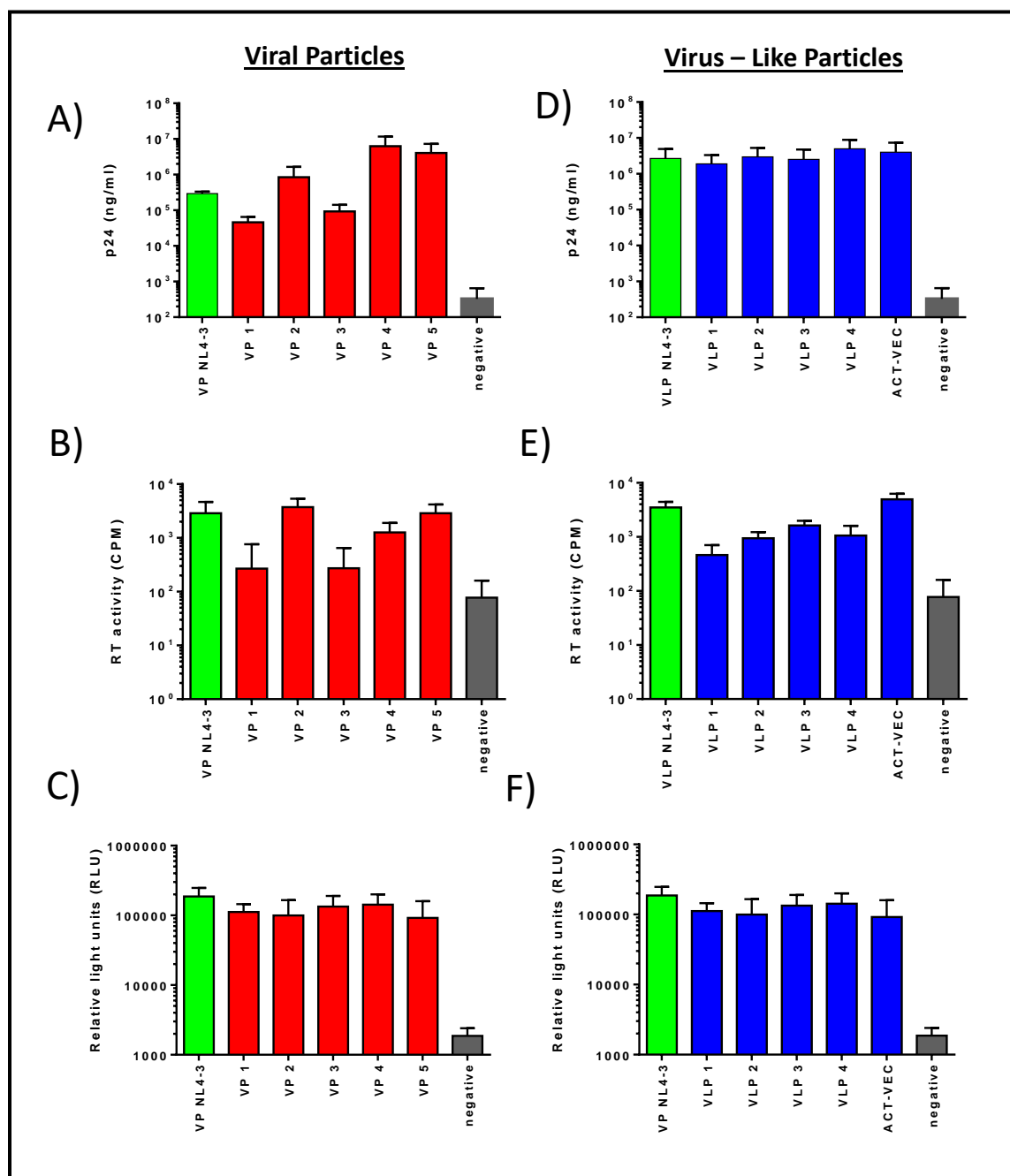
Figure 2-1: Viral particle (VP) and virus-like particle (VLP) formulations express similar viral protein concentrations.

pREC_nfl VP (red) and pREC_nfl_dS.1/mutIN VLP (blue) DNA constructs were used to transiently transfect 293T cells in 24-well tissue culture plates for 48 h. After 48h, culture supernatants were assessed for viral p24 production (**A** and **D**) using a p24 ELISA kit. Results shown represent mean p24 values (+/- SEM). Culture supernatants were also harvested to asses' VP and VLP reverse

transcriptase activity in counts per minute (CPM) using an in-house radioactive RT assay (**B** and **E**). To demonstrate the presence and functionality of HIV Env on VP and VLP, an HIV-1 co-receptor tropism assay was used (**C** and **F**). The 293T cells were transfected with the VP and VLP pREC-nfl plasmids and mixed with CD4$^+$/CCR5$^+$ U87 cells harboring the pDM128FLUC plasmid. Cell fusion elicits luciferase expression if the 293T cells express functional HIV-1 gp120/gp41 Env glycoproteins, Rev, and Tat from the pREC-nfl vectors. Results are represented by mean relative light units (RLU) (+/-SEM) with background luminescence subtracted from positive and negative results.

2.2.2 Vaccine VP and VLP are phenotypically identical to wild type virus and diverse

As described above, the dS.1/mutIN VLPs and wild type VPs contained similar amounts of Gag and Env proteins and similar levels of RT activity. However, the dS.1/mutIN VLPs were genetically formulated to 1) lack IN activity 2) to have reduced HIV-1 RNA, and 3) reduced ability to initiate and reverse transcribe (-) strand strong stop DNA. However, the general morphology of the VLPs compared to wild type HIV is unknown. Thus, we performed Transmission Electron Microscopy (TEM) on 293T cells transfected with our pREC_nfl constructs. All VP and dS.1/mutIN VLP pREC-nfl DNAs produced vesicular structures around the cells with some appearing to bud from the cell surface (Fig 2-2A). These circular structures contained electron-dense membrane layers and were ~100nm in diameter, akin to HIV particles. Tetherin, is constitutively expressed in restrictive human cells and cell lines such as HeLa, H9, Jurkat, Molt4, primary T cells, and primary macrophages[20]. As Tetherin activity is absent in the 293T producer cell line, we did not expect, nor did we observe any VP or VLPs "tethering" on the surface of transfected cells. However, since the full HIV-1 proteome is produced by transfection with pREC_nfl DNAs, we suspect that HIV-1 Vpu is produced and should down-regulate/degrade BST2/Tetherin[21] - supporting the use of the pREC-nfl as DNA vaccine vectors.

The size of the purified VP and VLP was verified by Dynamic Light Scattering (DLS). DLS depends upon Brownian motion and any resulting photon interference/deflection in liquid systems can be used to determine particle size and polydispersity. Following purification, the VP and VLPs had an average diameter centred around 100nm (Fig 2-2B), which agreed with the prior TEM measurements.
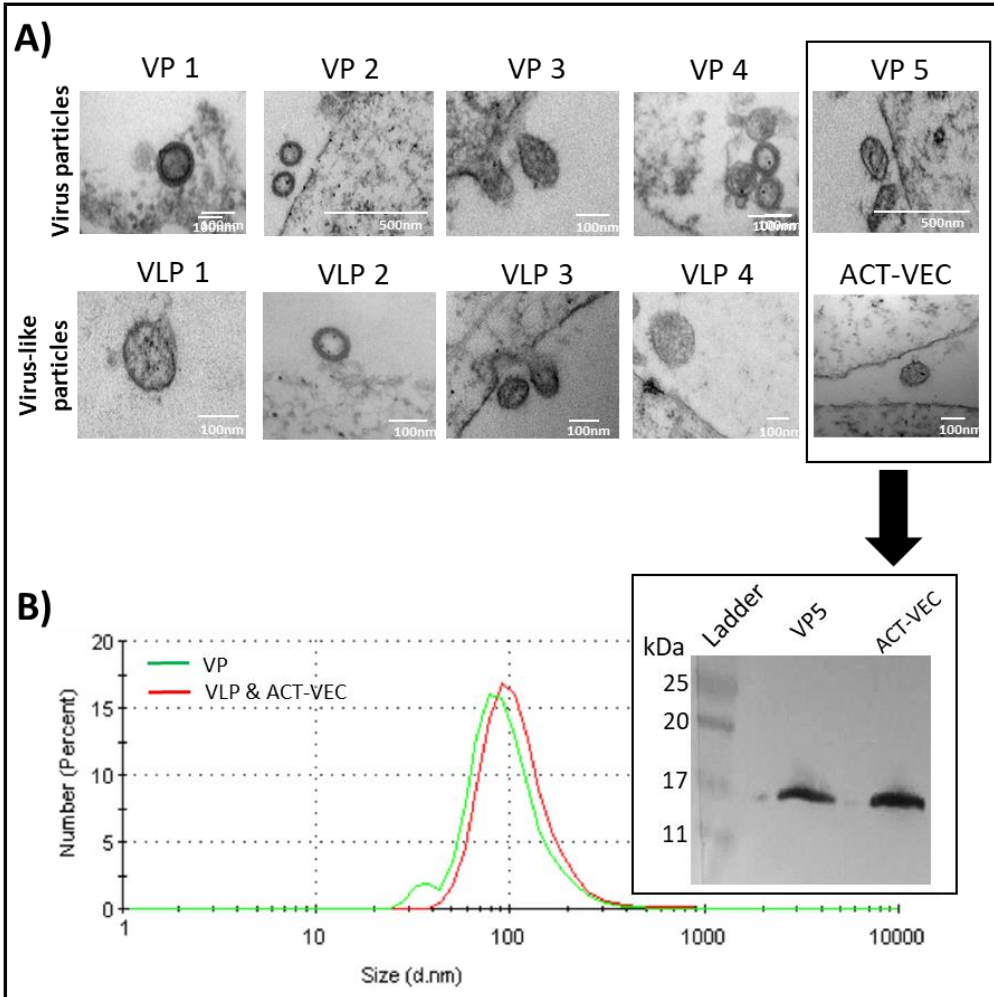
Figure 2-2: pREC-nfl derived VP and VLPs are morphologically similar to wildtype virus.

293T cells were transfected with pREC_nfl plasmids encoding VPs (- dS.1/mutIN) and VLPs (+dS.1/mutIN). Samples were fixed and embedded in Resin-Araldite Embed 812 before imaging via transmission electron microscopy (Philips CM10 TEM). Scale bar, is 100nm or 500nm (**A**). Purified VP and VLP preparations were analysed by dynamic light scattering at 25 °C using a Malvern Zetasizer Nano (Malvern Instruments Ltd). The intensity of the laser light scattered by the sample preparations was detected at 90° to the incidence beam. Data was analysed using Malvern software. (**B**).

2.2.3 Genetic diversity of patient-derived HIV-1 genomes with the pREC nfl vector.

The sheer genetic quasi-species of HIV-1 present within infected individuals, owing to a combination of low fidelity RT activity and genetic recombination, is a significant obstacle in the pursuit of viable therapeutic HIV vaccines. Thus, a successful vaccine might need to be sufficiently "rich in diversity" to have adequate protective coverage. Several vaccine studies have evaluated heterologous, polyvalent and sequential vaccine strategies to promote B cell affinity maturation and enhanced antibody production. Yeast-based recombination/gap repair is a highly efficient cloning technique and can yield hundreds of yeast colonies carrying pREC clones with HIV genomes. To determine the relative genetic bottlenecks in the cloning strategy, we RT-PCR or PCR amplified the C2-V3 env fragment from the patient plasma, from the recombined and purified pREC-nfl vector, and from the gRNA contained in the VLPs. These env PCR products were then subject to next generation sequencing (NGS) and analyzed using the methods described previously[22]. The dS.1/mutIN VLPs contain reduced gRNA, so we could not estimate genetic diversity within the patient derived VLP preparations. We did compare the genetic diversity within the VP pREC and subcloned dS.1/mutIN pREC by PCR amplification and NGS of the C2-V3 of Env. The topology of the phylogenetic trees is similar for VP pREC-nfl and sub-cloned dS.1/mutIN pREC-nfl for each patient (Figure 2-3). On average, each VP and VLP pREC_nfl contained 65 (range = 49 - 98) and 30.6 (range = 22 - 29) unique sequences, corresponding to an average of 0.006 (range = 0.00192 – 0.022) and 0.0178 (range = 0.0013 – 0.082) substitutions/nucleotide (Table 2-7S).
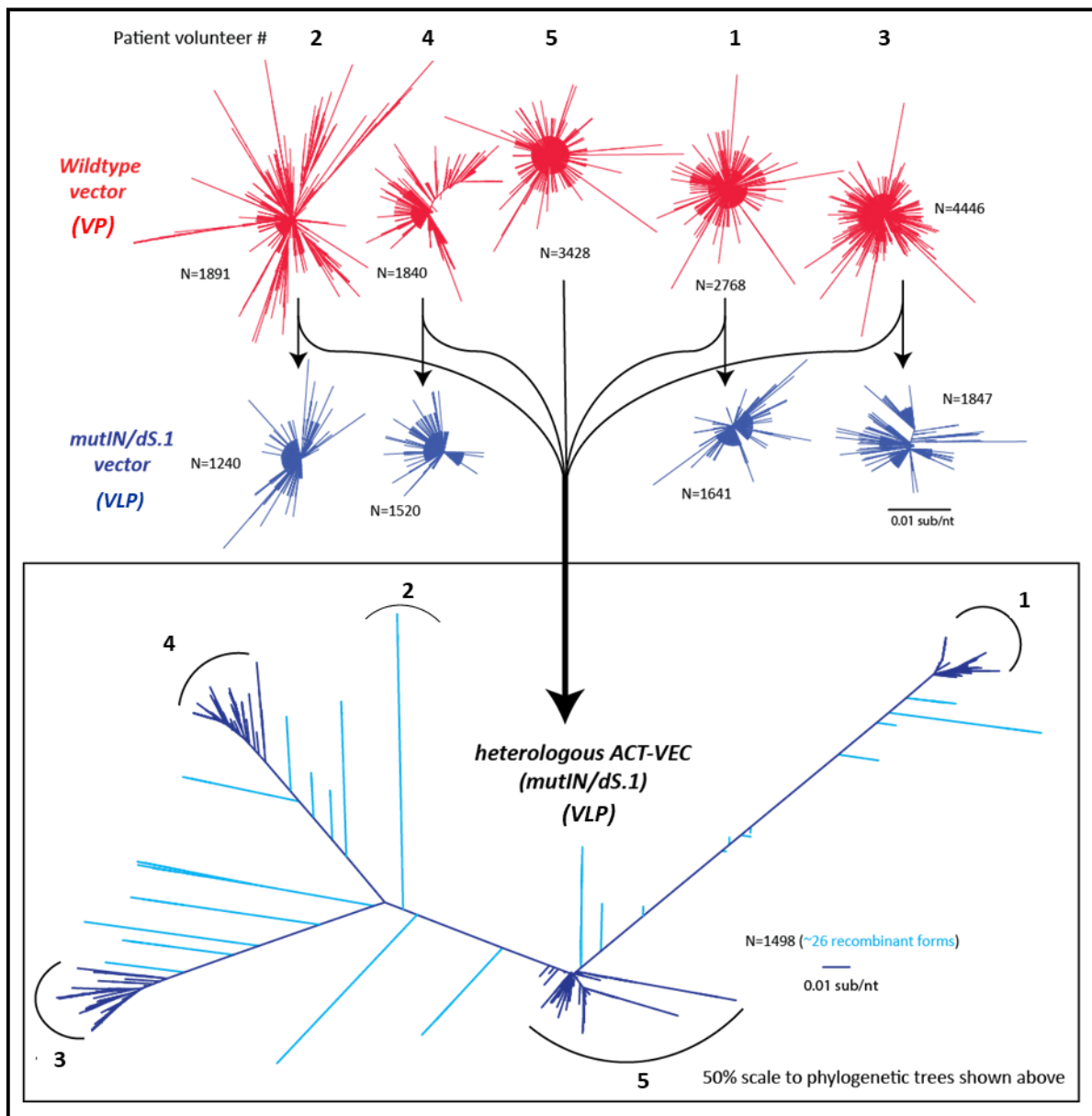
Figure 2-3: VPs and VLPs are genetically diverse preparations.

Neighbor joining trees of nucleotide sequences were generated with MEGA6 and visualized with FigTree 1.4.2 to highlight sequence heterogeneity. Phylogenetic trees were reconstructed for viral particles (red) and for virus like particles (blue). VLP pREC_nfl DNAs were combined to generate Het_B_ACT-VEC.

2.2.4 Reducing genomic RNA packaging and inactivating integrase

Full genome amplifications of the 5 patients were repeated but using primer sets to disrupt the gRNA packaging signal and/or the integrase active site (Figure 2-6S). The 5' primer of fragment 1 contained either 9 point mutations in stem loop 1 (698C>T, 718C>G, 719G>T, 720G>C, 721C>G, 722A>T, 723A>T 724G>C and 731G>A, herein designated dS.1) or a 33 bp deletion within stem loop 3 (nucleotides 755-787, herein designated ΔSL3) as described previously by Clever *et al.,* 1997[23]. This work was initially carried out to identify the minimal gRNA packaging element of HIV-1 using HXB2 pseudotyped with amphotropic murine leukemia virus. However, in our system it was necessary to confirm that these mutations would reduce gRNA encapsidation in our patient-derived VLP. Thus, our VLPs would be inherently safer vaccine constructs compared to our VP formulations. Initially, the dS.1 mutation and ΔSL3 deletion was introduced into the pREC-nfl$_{NL4-3}$ and used to transfect 293T cells (Figure 2-7S A, C and E). Resulting particles were harvested and purified from cell free supernatants and found to have 142- and 11.6-fold less gRNA with the dS.1 and ΔSL3 mutations, compared to NL4-3-viral particles. This work was further verified using HIV-1 clade C Env 1086 (Figure 2-7S B, D and F). We then mutated the patient-derived viruses, VP3, VP4, and VP5 and again found that the dS.1 mutations impaired gRNA packaging more so than the ΔSL3 mutations (Fig 2-4A & Figure 2-8S C).

   With evidence that dS.1 reduced gRNA packaging in primary vaccine particles, we proceeded to introduce both the dS.1 mutation and the RRK>AAH integrase mutations via primer-related replacement during PCR amplification of the 5'/upstream genome half and the integrase mutations into the 3'/downstream half of the genome. The two overlapping PCR products representing the halves of the genome with these mutations were then cloned into our pREC vector by yeast recombination/gap repair as described above. Following plasmid purification and particle production from transfected 293T cells, we tested these dS.1/mutIN mutated VLPs for CAp24/Gag content by ELISA and Western Blot, encapsidated gRNA by real time RT-PCR, endogenous RT activity using exogenous poly(rA)oligo(dT) template or the endogenous gRNA template (Figure 2-1), and finally, integrase activity by using VLPs soaked in lysis buffer containing radiolabelled substrates for dinucleotide cleavage by IN (data not

shown). The addition of the RRK>AAH IN mutations, removal of the 5'LTR, and introduction of the dS.1 mutations did not impact Gag/CAp24 levels or RT activity on an exogenous template in the VLP preparations. However, none of these mutated VLPs had dinucleotide cleavage activity/end processing as mediated by functional IN, as observed and compared with wild type HIV particles (data not shown).

Finally, we repeated the cell-to-cell fusion assay by transfecting 293T cells with the pREC-nfl vectors harboring the mutations with the patient-derived sequencing. Evidence of wild type cell fusion suggest that Env is expressed from pREC and can bind to CD4$^+$/CCR5$^+$ on the U87 cells. Western blots show the presence of Env in cell-free supernatant. Previous studies using a Vpr-bla construct expressed in trans and encapsidated into VLPs[24], clearly show VLP entry into CD4$^+$/CCD5$^+$ cells and that the levels of VLP entry correlated with the levels of cell-to-cell fusion mediated by pREC-nfl (used to produce the same VLP).
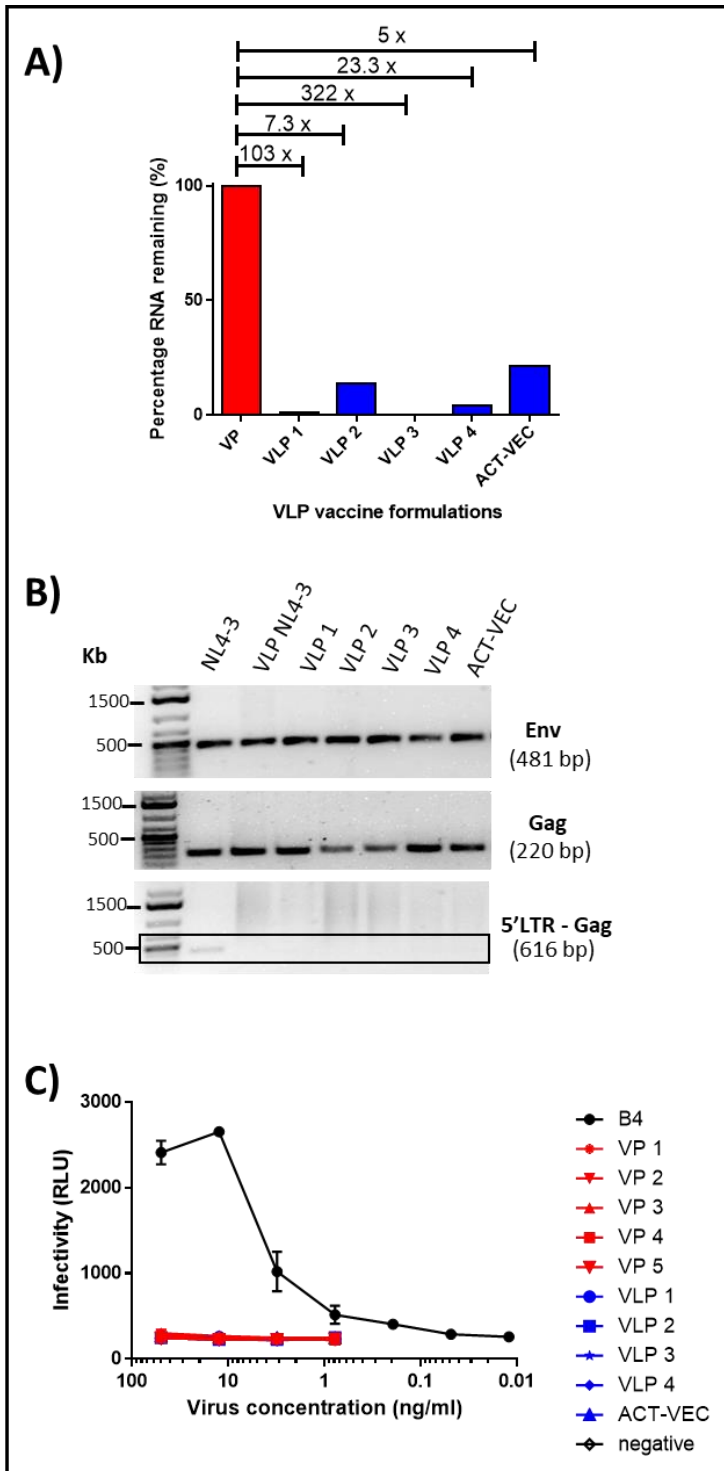
Figure 2-4: Virus-like particle formulations are non-infectious due to engineered RNA packaging defects and deletion of the HIV-1 5' LTR.

RNA packaging knockdown in individual VLP formulations (VLP1-4 + Het_B_ACT-VEC) were compared against near-full length viral particles (VP) formulations lacking mutations in the RNA packaging sequence by first isolating viral RNA and then by qRT-PCR using a gag primer set (**A**). The VLP encoding pREC_nfl DNA's were evaluated for the presence/absence of HIV-1 gag, env and 5' LTR by PCR and gel electrophoresis using gag, env and 5'LTR – gag primer sets. Samples derived from the same experiment and gels were processed in parallel (**B**). VP (-dS.1/mutIN) and VLP (+ dS.1/mutIN) formulations were compared to infectious B4 virus for infectivity using luciferase expressing TZM-bl cells (**C**). Infectivity results are represented by relative light units (RLUs). luciferase quantification in a Synergy H4 Hybrid microplate reader using 50 μl of luciferase assay reagent.

2.2.5 VLPs are non-infectious, devoid of 5'LTR and have reduced viral RNA packaging

While inactivated (killed) whole virus particles have been used to prevent a wide range of viral diseases, the use of AT-2 inactivated, UV-irradiated whole HIV particles as a vaccine has been a concern due to safety but has recently undergone a phase I clinical trial evaluation showing no residual vaccine replication or any evidence of vaccine viral genetic material[25]. Given the enhanced safety considerations in our preparation, it is important to note that previous generations of the VPs have been tested in mice, rabbit, and macaques with no adverse effects noted. Furthermore, the new generation of VLPs are currently being prepared for phase I clinical trials. Both the VP formulations and the dS.1/mutIN VLPs were unable to infect and replicate in a permissive luciferase expressing TZM-bl cell line. This contrasts with B4, an infectious subtype B chimeric virus (Env from a primary isolate placed into an NL4-3 backbone), which was readily able to infect this highly susceptible cell line in a concentration dependent manner (Fig 2-3C).

2.2.6 Both VP and Het_B_ACT-VEC can stimulate antigen-specific memory T cell responses.

The five volunteer-derived dS.1/mutIN pREC-nfl plasmid DNAs were all combined to generate a highly heterogeneous and polyvalent Het_B_ACT-VEC VLP preparation. This was used to transfect 293T cells and produce Het_B_ACT-VEC VLP formulations. We PCR amplified and sequenced the VP, dS.1/mutIN (VLP) and Het_B_ACT-VEC pREC-nfl sequences using NGS. Phylogenetic trees reveal a topology similar to a tree containing the population of all other pREC-nfl as expected (Figure 2-3).

To determine whether VP and Het_B_ACT-VEC were antigenic and capable of stimulating antigen-specific T cell recall responses, we assayed the formulations in a MDDC – CD4+ T cell co-culture assay using cells derived from HIV infected volunteers (Figure 2-5A). PBMCs from seven volunteers were purified by negative selection to generate isolated, untouched CD4+ T cells with purity >95%. Patient derived MDDCs were grown by plastic adherence and in the presence of IL-4 and GM-CSF for 6 days. Resulting MDDCs were checked for phenotypic markers of differentiation such as HLA-DR, CD83 and CD209 (Figure 2-9S B). MDDC were pulsed overnight with VP 5 or Het_B_ACT-VEC before washing and co-culturing with autologous CD4+ T cells in a human IFN-γ ELISpot assay (Figure 2-5). As shown, the VP 5 (**p<0.005) and the Het_B_ACT-

VEC (*p<0.05) were antigenic and generated significant numbers of SFU/10^6 CD4$^+$ T cells when compared to the unstimulated MDDC-CD4$^+$ T cell co-cultures (Figure 2-5B). No statistical difference in the generation of SFU was observed between VP 5 and the Het_B_ACT-VEC formulation (p>0.05). Thus, demonstrating that the Het_B_ACT-VEC vaccine construct is antigenic and can stimulate memory CD4 T cell recall responses in primary human cells. We further verified the ability of Het_B_ACT-VEC and VP's to stimulate primary and secondary immune responses using our MDDC-CD4 T cell co-culture assay and PBMC from healthy donors using intracellular cytokine staining flow cytometry (Figure 2-9S A-B). In this instance, Het_B_ACT-VEC was able to induce TNF-α and IL-2 cytokine responses (average 2-fold increase over media control) with only a low-level increase in IFN-γ. The VPs tested, especially VP2,4 and 5 were also capable of eliciting primary CD4 T cell responses with the magnitude greater than that seen with Het_B_ACT-VEC. Again, no statistical difference in the generation of cytokine responses was observed between the different VP's and the Het_B_ACT-VEC formulation was detected (Figure 2-9S B).
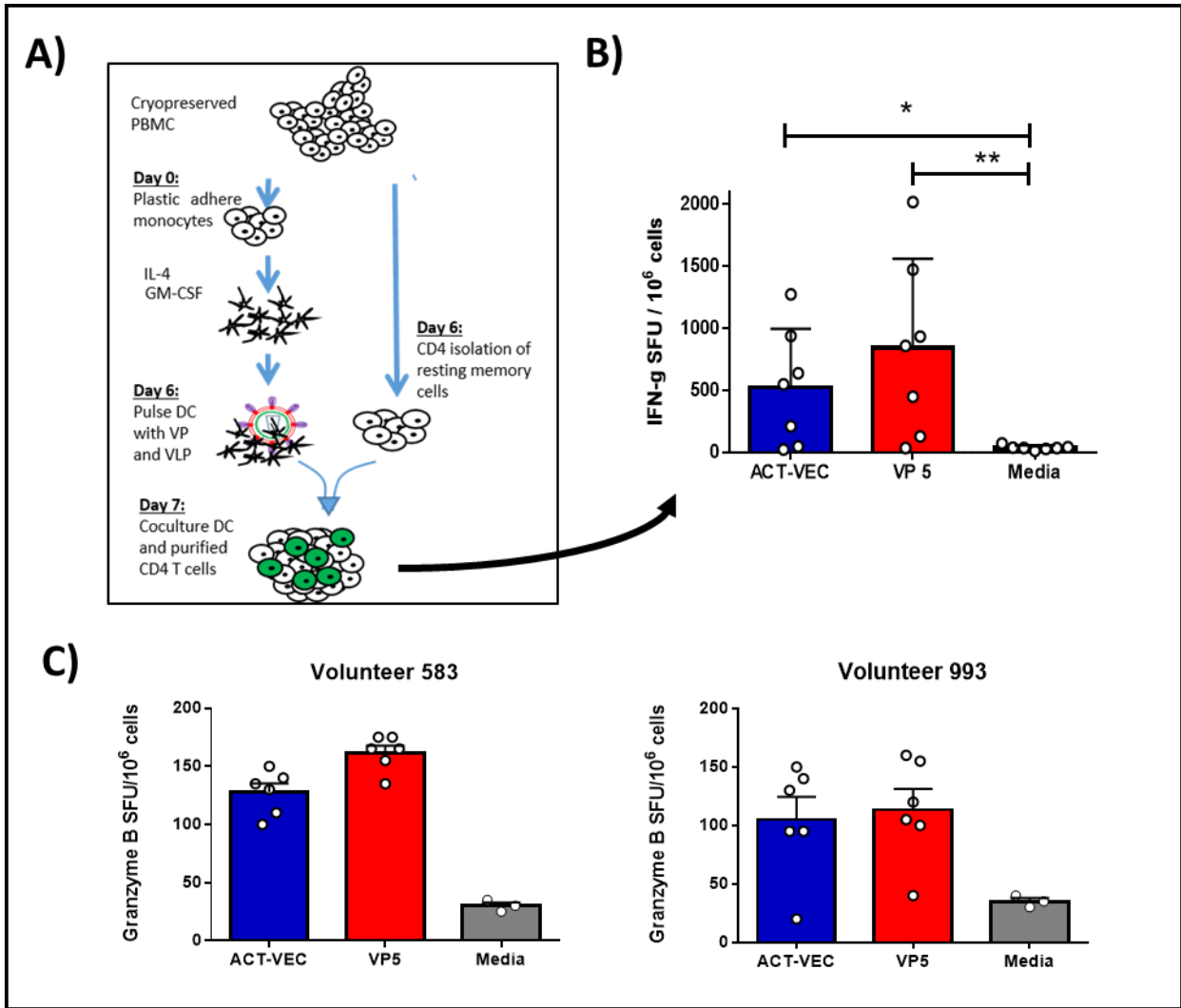
Figure 2-5: Purified VP and Het_B_ACT-VEC formulations are capable of human CD4+ T cell activation in vitro.

PBMC from fully consented HIV$^+$ volunteers were used to generate monocyte derived dendritic cells (MDDC), which were pulsed overnight with Het_B_ACT-VEC VLP or VP 5 and co-incubated with autologous purified CD4$^+$ T cells (**A**). Cells were cultured overnight in a human IFN-γ ELISpot assay and the IFN-γ spot forming units were enumerated per 10^6 CD4$^+$ T cell using ImmunoSpot S5 UV Analyzer and ImmunoSpot 5.0.9 software (**B**). Results shown are mean SFU/10^6 CD4$^+$ T cells (+/- SEM). Graphics depicted in this figure were generated by the authors.

2.3 Discussion

Over the last few centuries, empirically derived vaccines have slowly been replaced by rationally designed vaccines. During this time, killed/attenuated vaccines have demonstrated huge success in providing protection from a range of communicable diseases. However, safety concerns regarding insufficient attenuation and inactivation processes still remain. In the context of HIV-1 vaccines, attenuated versions of HIV raises serious ethical concerns due to the fear of reversion to virulence following vaccination, or the potential for recombination with resident HIV following therapeutic vaccination[26,27]. Methods to inactivate HIV-1, such as AT-2 treatment and UV irradiation, may result in a safe vaccine[25], but these conditions may also alter antigenicity and could affect the conformation of viral glycoproteins. As such, research into safer and stronger alternatives to the killed/attenuated HIV-1, has gained significant traction. This study describes the development of non-infectious VPs derived from infected patients, and their reformulation into VLPs that are then used to produce a heterogeneous VLP formulation called Het_B_ACT-VEC. The Het_B_ACT-VEC is currently being tested as a therapeutic vaccine. In the latter case, the Het_B_ACT-VEC is being used as a latency reversal agent as part of the "Shock and Kill Strategy", the aim being to trigger proviral transcription and the elimination of infected cells[15,28].

Our first prototype of this HIV-1 vector, termed VP, was derived from the pREC-nfl construct[17]. HIV-1 mRNA is transcribed off pREC-nfl (near full length) from a minimal CMV promoter, deleting the 5'LTR promoter/enhancer region such that transcription is initiated at the first nucleotide of the primer binding sequence. Aside from deleting the Tat-transactivating response element (TAR) found within the 5'LTR, all other RNA regulatory sequences remain intact such that all HIV-1 mRNA species (un-spliced, singly spliced, and multiply spliced) are expressed, the full HIV-1 proteome translated, and virus particles assembled and released from these cells. We have previously shown that the nfl virus particles released from this system cannot initiate reverse transcription (i.e. no minus strong stop DNA synthesis), are non-infectious, and cannot be complemented by superinfection with a wild type HIV-1[17].

Despite rendering the lentiviral VPs non-infectious, the presence of nfl viral genomic RNA within vaccine formulations still poses a concern in vaccine development. Therefore, we

introduced extensive mutations into the stem loop 1 (dS.1) of the RNA packaging element which strongly reduced genomic RNA encapsidation in VLPs, paralleling observations made by Clever and Parslow with a lab-adapted HIV-1 clone[23]. In our studies, the dS.1 mutations coupled with the deletion of the 5'LTR did not impact p24 content or RT activity in the VLPs but depleted gRNA encapsidation and resulted in non-infectious particles. Finally, during the amplification of the patient-derived HIV-1 genomes, we introduced a $_{262}$RRK>AAH mutation into the active site of HIV-1 integrase. In contrast to the nfl VPs, the dS.1/mutIN VLPs and Het_B_ACT-VEC (with Δ5'LTR/mutIN/dS.1) harbored a defective IN incapable of the dinucleotide cleavage, which ensured the nullification of viral infectivity.

In the absence of Gag-gRNA complexes, Gag can still oligomerize to produce virus-like particles of varying size and structure whereas the inclusion of the full proteome (including the Env glycoprotein) appears to generate more uniform virus particles, which was evident from our electron microscopy images on all formulations. While HIV viral particle formation is dependent only on Gag production, Env is required for virus entry into host cells and could elicit protective antibody responses. We propose that these fully processed wild type virus particles may be highly immunogenic and could induce neutralising responses, considering the viral proteins should be in a native "wild type" structure, and can even undergo conformational changes related to viral entry, core deposition and dissolution in the cell cytoplasm. However, it should be pointed out that not all VPs or VLPs will carry intact, native-like trimers, nor will all the expressed Env become appropriately cleaved, and a fraction would certainly exist as monomers, dimers and gp41 stumps[29]. The development of a neutralizing antibody response would of course be highly beneficial for an anti-HIV prophylactic vaccine, enabling the neutralization of transmitted founder viruses at the earliest time points following virus exposure; however, anti-HIV antibody and neutralizing antibody responses would also be beneficial for therapeutic vaccines. In the latter case, it is envisaged that either an elicited neutralizing or antibody-dependent cell cytotoxicity response could aid therapeutic vaccination by blocking de novo infections occurring in response to latency reversal (Shock) and also in the eradication (Kill) of virally infected cells.[15,28]

While B-cell immunogens are important in providing protection through the generation of neutralizing and non-neutralizing antibody production, T-cell immune responses play an important role in controlling viral loads and priming B-cell responses. Therefore, it stands to reason that an efficacious therapeutic vaccine should elicit both T- and B-cell responses to either prevent infection at the level of the mucosa or eliminate infection through sterilizing immunity. Hence, it is important that our formulations can elicit both CD4 T helper cell and GzB cytotoxic functions in PBMC. Until such time that a broadly neutralizing antibody response can be successfully elicited through vaccination, anti-HIV cytotoxic responses could be highly beneficial in therapeutic vaccine settings, i.e in controlling/eradicating productively infected cells within HIV-infected individuals[15,30,31]. Attenuated viral vectors (e.g., ALVAC or Ad5) expressing more conserved HIV-1 proteins (e.g., Gag) or protein motifs (e.g., repeated strings of immunodominant/conserved HIV peptides) have been tested as CTL-based vaccines with moderate success in animal models but mostly failed in human clinical trials[32]. Although many factors led to these vaccine failures, CTL escape mutations rapidly appear in the infecting HIV during both preventative and therapeutic vaccine trials. These studies, as well as the failure of most humoral based vaccines, have prompted us to construct and test a heterogenous HIV vaccine, which consist of "dead" HIV-1 particles lacking undesirably foreign vector components.

Our yeast recombination system produces vectors that harbor the same HIV-1 quasi-species diversity found within patient samples. By combining the subtype B HIV quasi-species from 5 patients, we have generated a heterogenous dS.1/mutIN VLP (termed Het_B_ACT-VEC). To further develop our therapeutic and cure based vaccine strategies, we screen patients for subtype infection and then employ the respective ACT-VEC, currently being tested in *ex vivo* experiments with the aim of selecting the most effective vaccine modalities for use in a future clinical trial.

We are currently testing different heterogenous forms of this dS.1/mutIN VLP as an anti-HIV therapeutic/cure strategies, designed to elicit T cell responses for activating the latent HIV pool and for reducing viral loads. The DNA vector pREC-nfl is also being tested as a DNA vaccine administered by intramuscular electroporation in animal models, and may be very effective in combination with the VLP and Het_B_ACT-VEC for prime-boost therapeutic

vaccinations. As described in a recent study, Het_B_ACT-VEC may also lead to latency reversal and elimination of much of the HIV-1 reservoir that remains in these HIV-infected patients receiving cART. In conclusion, our dS.1/mutIN VLP formulations appear safe *in vitro,* and we anticipate that VLPs and Het_B_ACT-VEC formulations will be safe for use in humans; however, this will need to be confirmed in future planned non-human primate (NHP) studies and human clinical trials. The VLPs and Het_B_ACT-VEC also present the entire HIV proteome in the correct conformation, reflect the HIV-1 genetic diversity within infected individuals and consequently, have wide range of uses.

2.4 Materials and methods

2.4.1 Ethics statement:

For antigenicity studies, either HIV-positive volunteers were recruited from the HIV adult clinical St Mary's Hospital (Imperial College NHS trust), through a protocol approved by the NHS Health Research Authority (protocol number: 14SM1988) or healthy volunteer PBMC were purchased from Canadian Blood Services under institutional REB approval (no: 106951). Written informed consent was provided by all HIV+ study participants prior to the study start. The peripheral blood mononuclear cells (PBMC) from HIV+ volunteers used in these studies had suppression of viremia to <50 copies HIV-1 RNA/ml for > 6 months on ART. For viral particle (VP), virus like particle (VLP) and Het_B_ACT-VEC production, HIV+ sera from five consenting HIV+ adult volunteers were obtained under internal review board approval (AIDS125) at Case Western Reserve University, (CWRU, USA). Methods were performed in accordance with relevant regulations and guidelines.

2.4.2 Viral Particle (VP) and Virus-like particle (VLP) vaccine production:

All formulations were cloned using a similar protocol to that previously described and schematically depicted in Figure S1[16–18]. Briefly, sera-derived viral RNA was isolated using a viral RNA isolation kit (Qiagen, USA) and reverse transcribed to cDNA (Agilent Technologies, USA) using two primers to generate a 5' (5020R) and 3' (1.R3.B3.R) fragment encompassing the entire HIV-1 genome. The two overlapping cDNA fragments were then PCR amplified in a nested PCR protocol using 5' and 3' primer pairs described in Table S1. The two fragments were

then transfected into *Saccharomyces cerevisiae* in a 1:1 ratio with 2 μg SacII linearized plasmid, pRECΔgag-U3/URA3. Yeast colonies were selected on complete medium lacking leucine (C–Leu) plates supplemented with fluoroorotic acid (FOA). The resulting plasmid vectors were isolated by an in-house yeast miniprep and used to transform bacteria to amplify the DNA plasmid for purification as described previously. It is important to note that the PCR products harbored the amplified patient quasispecies, and as such, >100 yeast colonies were removed from Leu–/FOA plates for bulk plasmid purification and eventual reconstitution of sample quasi-species. The resulting plasmid constructs were isolated and then then used to transfect 293T cells with Fugene 6 transfection reagent (Promega, USA) to produce viral VP and VLPs. This procedure of highly efficient yeast-based recombination/cloning followed by 293T transfections is believed to preserve the HIV-1 quasi-species population better than a similar approach using bacterial restriction enzyme cloning. VP and VLPs were then purified by centrifugation through 100 KDa MWCO centrifuge tubes (Amicon, USA) and re-suspended in sterile PBS.

2.4.3 Vaccine quantitation and protein production assessment

VP and VLP production from 293T cells was monitored for transfection efficiency by p24 ELISA assay, provided under an MTA by the AIDS Vaccine Program, National Cancer Institute (NCI) at Frederick, MD, USA. A radioactive reverse transcriptase (RT) assay was also used to measure VP and VLP levels in cell-free supernatants as described previously[18]. Viral proteins in formulations were also analyzed by Western Blot. using NuPAGE™ Novex™ 3-8% Tris-Acetate Protein Gels (Thermo-Fischer Scientific) and a 1:100 dilution of heat-inactivated serum derived from SHIV infected macaques, before addition of a 1:2000 dilution of goat anti-monkey IgG: HRP (Bio-Rad). Samples were then developed with DAB SK-4100 (Vector Laboratories).

2.4.4 Size estimation of vaccine particles

VP and VLP size and particle distribution were measured using dynamic light scattering with a Malvern Zeta-Sizer Nano (Malvern Instruments Ltd) at 25 °C. Briefly, purified VP and VLPs were diluted into 1ml PBS and placed into 4.5 ml polystyrene analysis cuvettes (Fisher Scientific, Ca). The intensity of laser light scattered by the sample preparations was measured at 173° to the incident beam. The data were analyzed using proprietary Malvern software, DTS (Nano Version

5.0), supplied with the machine. The size distribution and the polydispersity were measured using non-invasive back scatter (NIBS).

2.4.5 Next generation sequencing analysis of vaccine formulations

The C2-V3-C3 region of envelope was amplified by an external-nested PCR amplification using the primers forward EnvB and reverse ED14 (external) and forward E80 and reverse E125 (nested) using PCR cycle conditions as described previously[33]. To prepare the amplicon library for 454 sequencing, fusion primers including the Roche 454 titanium key sequence, a multiplex identifier (MID) sequence for forward and reverse primers, followed by the template specific forward (E110) and reverse (E125) sequences were generated. The nested products were re-amplified with barcoded MIDs. The PCR products were run on a 1% agarose gel to verify the 406 bp size then purified with the Agencourt AMPure XP bead system with a bead : DNA ratio of 0.7:1 according to the Roche manual. Following purification PCR amplified sample libraries were quantified using the Quant-iT PicoGreen ds DNA assay kit (Invitrogen), diluted and pooled together at 10^6 molecules/μl for pyrosequencing as per Roche 454 instructions.

Following emulsion PCR (emPCR) at a ratio of 0.5 molecules of sample library per bead, 5×10^5 enriched beads were loaded onto the titanium picotiter plate which was run on the Roche 454 GS Junior instrument. Raw sequence data were extracted by the MID tag using a custom analysis pipeline. The 454 amplicon adapters were trimmed and sequences of less than 200bp were discarded. Sequences were edited using BioEdit v7.2.5 and aligned using maximum likelihood methods (MUSCLE)[34,35]. Neighbor joining and maximum likelihood trees were constructed with SEAVIEW 4 and visualized with FigTree 1.4.2. Kimura genetic distance analysis within each sample were calculated using MEGA 6 and is expressed as substitutions per nucleotide (s/nt)[36,37]. Any genetic variants with hypermutations at homopolymeric tracts and/or appearing less than 3 times were removed from the analyses.

2.4.6 Isolation of resting CD4^{+} T lymphocytes and Monocyte Derived Dendritic Cells.

CD4^{+} T lymphocytes were enriched from peripheral blood mononuclear cells (PBMC) by negative depletion (Miltenyi Biotec) using magnetic microbeads. To obtain immature DCs,

PBMC were plastic adhered at 37 ˚C for 2 h. Adhering monocytes were washed to remove non-adherent cells then differentiated into monocyte derived dendritic cells (MDDC) by culturing in complete RPMI (10% FCS + 2 mM L-Glutamine) supplemented with GM-CSF and IL-4 (1,000 and 500 U/ml respectively) for 6 days.

2.4.7 TZM-bl infectivity assay

The infectivity of viral particle and VLP preparations were estimated in TZM-bl cells by luciferase quantitation of cell lysates (Promega, Madison, WI). Briefly, TZM-bl cells were seeded at 1×10^4/well prior to addition of 50 ng/ml (based on p24) viral particles and VLP formulations. After 48h incubation, cells were washed with PBS and lysed with 100 µl of lysis reagent. A 50 µl volume was used for luciferase quantification in a Synergy H4 Hybrid microplate reader (BioTek Instruments, Inc., Burlington, VT) using 50 µl of luciferase reagent. The extent of luciferase expression was recorded in relative light units.

2.4.8 Transmission Electron Microscopy

VP and VLP transfected 293T cells were collected in 15ml tubes before pelleting at 1250 rpm for 10 min. Cells were washed with sodium cacodylate (pH 6.5) before re-suspending in 500ul of 2.5% glutaraldehyde in sodium cacodylate. Supernatants were removed, and pelleted cells re-suspended in 1% osmium tetroxide in sodium cacodylate for 1 h with shaking. Samples were then centrifuged and washed with deionized water. Dehydration was performed by re-suspending samples in 1 ml of increasing concentrations of acetone (30%, 50%, 70%, 90%, 95%, 100%) for 10 min each. Serial resuspensions with acetone : TEM Resin-Araldite EMbed 812 (2:1, 1:1, 1:2, whole TEM resin) followed, until all acetone was replaced by resin. Samples were then baked at 60 °C for 48 h and resin embedded samples cut into 70 nm wide sections using an UltraCut UltraMicrotome (Sorvall) before copper mesh mounting and staining with Uranyl Acetate. Samples were dried and stained with Lead Citrate before washing thoroughly with sterile water. Samples were air dried then imaged on a Philips CM10 TEM.

2.4.9 Immunogenicity assays

Human IFN-γ enzyme-linked immunosorbent spot (ELISpot) assays (Mabtech, USA) and intracellular cytokine staining flow cytometry were carried out on MDDC-CD4$^+$ T cell co-cultures. ELISpot were carried out as per the manufacturers instructions. Briefly anti-IFN-γ pre-coated plates were washed with sterile PBS and then blocked for 30 min using complete RPMI. Plates were again washed before addition of 1×10^6 cells/ml CD4$^+$ T cell. The MDDC stimulated CD4$^+$ T cells were then incubated for 16 h to assess the number of HIV-specific CD4$^+$ T cells. Unstimulated and 5 µg/ml phytohemagglutinin (PHA)/ionomycin (Iono) (Sigma, USA)-stimulated cells served as controls. To detect spots, biotinylated anti-IFN-γ antibody was added at 1 µg/ml for 2 h before washing and incubating with streptavidin-HRP for 1 h. Plates were washed and 100 µl/well of TMB substrate was added. Spot forming units (SFU) were enumerated per 10^6 CD4$^+$ T cell using ImmunoSpot S5 UV Analyzer (Cellular Technology Ltd., Cleveland, OH) and ImmunoSpot 5.0.9 software. Results are mean values (+/- SEM). For flow cytometric analysis of T cell activation, CD4$^+$ T cells, were incubated for 2 h with ACT-VEC or VP pulsed MDDCs before 4h incubation with, monensin. Samples were washed using FACs buffer (2.5% FCS in PBS) and surface stained with anti-CD3, anti-CD4 and anti-CD69 antibody. Samples were washed again before permeabilising using a BD FACS Fix Perm kit (Becton Dickinson, USA). The samples were then incubated with anti-IFN-γ, anti-TNF-α and anti-IL-2 antibody in FACS perm wash solution before washing in FACs buffer and then fixing in 1.5% methanol-free paraformaldehyde (Polysciences, USA) in PBS. Samples were analyzed on a FACS LSR II instrument with FACS Diva software. Data analysis was performed with FlowJo (Treestar Inc., OR, USA).

2.5 Acknowledgments

2.6 Competing Interests

The authors declare no conflicts of interest.

2.7 Author Contributions

JFSM, JP, RS, DC, SF, and EJA designed experiments. JFSM, YG, KK, TB, and JP built the VP, VLP and ACT-VEC constructs. JP, KK, JFSM performed all qRT-PCR experiments and analysis. KK, KB and JP performed deep gene sequencing and both JFSM and KB performed all the radioactive RT assays. KK and EJA performed NGS analysis. JK and JP did Veritrop assays. JP did the zeta sizing and electron microscopy work. SF, RS, PM and DK, recruited, consented and processed blood samples. JFSM, KK and JP did the immunogenicity studies and JP, KK, JFSM and EJA wrote the manuscript. The manuscript was reviewed by the authors.
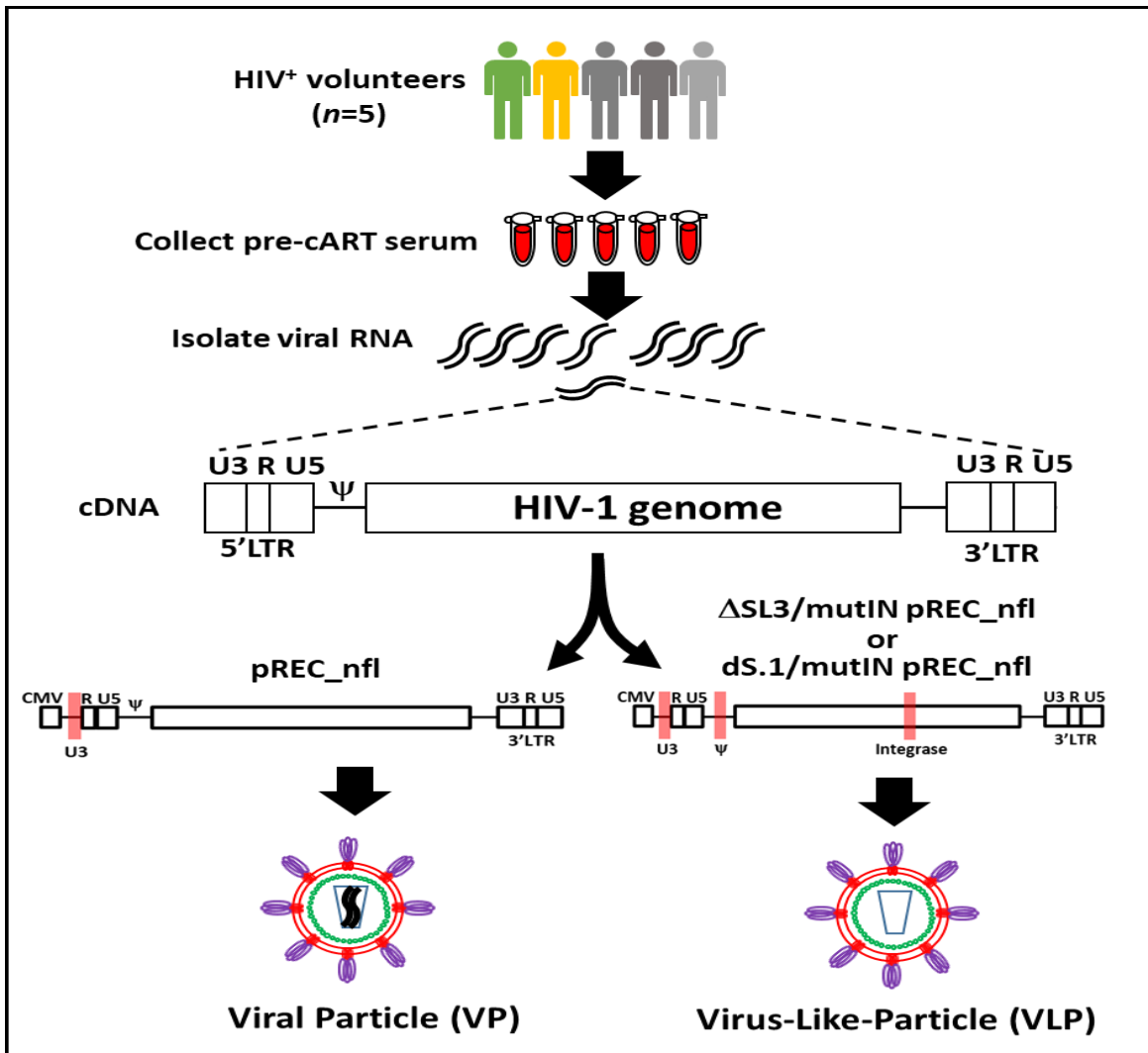
Figure 2-6S: Schematic of the VP and VLP cloning protocol

Schematic representation of the VP and VLP cloning strategy. Serum from consented HIV+ volunteers was used to extract viral RNA and generate two overlapping cDNA fragments. The cDNA is then used in a nested PCR reaction to generate two overlapping fragment of viral DNA for recombination in yeast using our in-house developed pREC_Δgag-U3 recombination vector. In the nested PCR, mutagenic primers dS.1 or ΔSL3 insert extensive nucleotide substitutions in stem loop 1 of the RNA packaging sequence or delete stem loop 3. Successful recombination in yeast and subsequent bacterial amplification results in pREC_nfl or ΔSL3/mutIN pREC_nfl or dS.1/mutIN pREC_nfl DNA for use in VP and VLP production. Areas where modifications to the

viral genome were introduced are indicated by red shading. Graphics depicted in this figure were

generated by the authors.
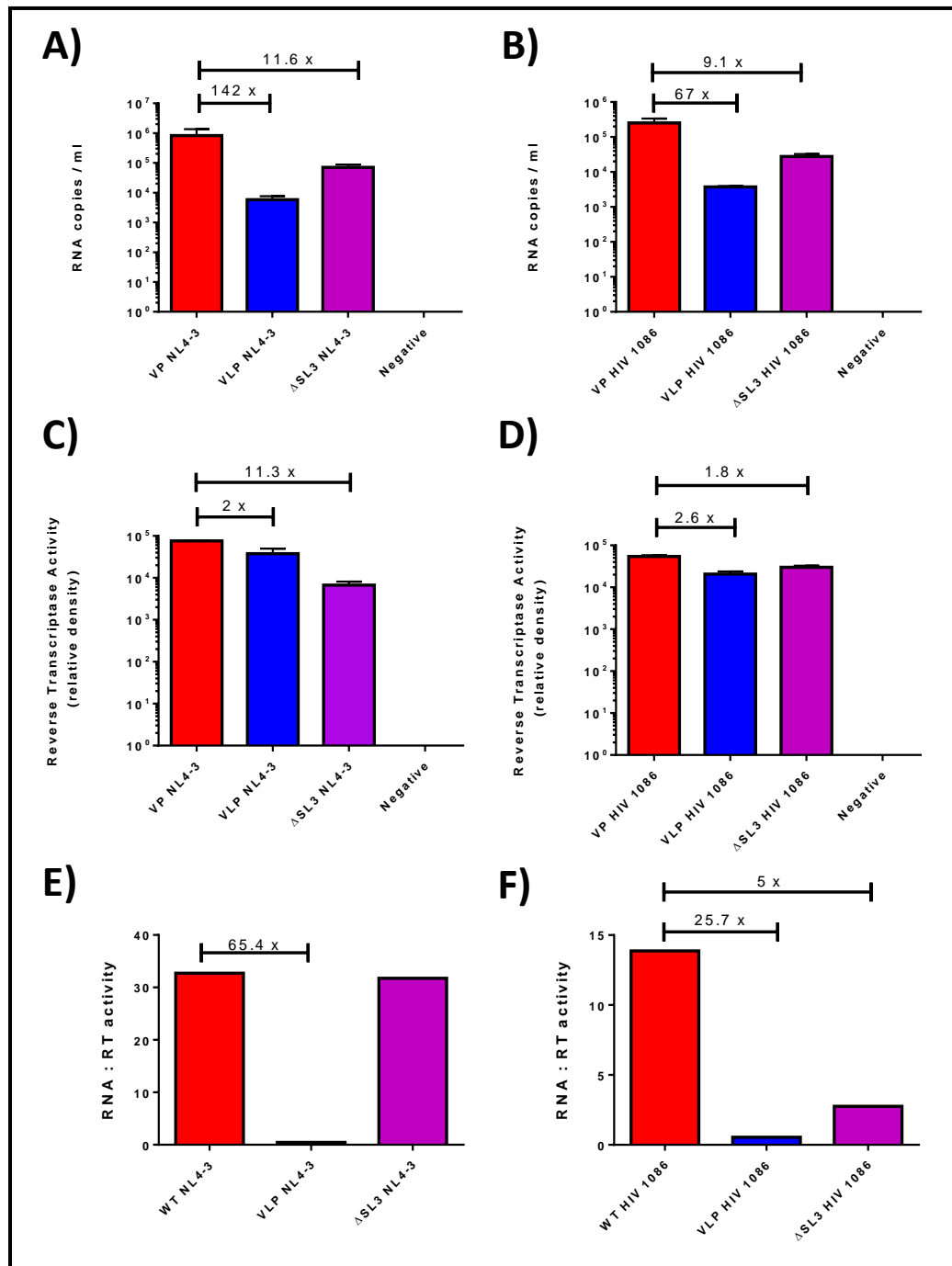
Figure 2-7S: Comparison of the impact on RNA packaging by differently engineered RNA packaging mutations.

pREC_nfl plasmid constructs using NL4-3 and HIV 1086 as the vector backbone were constructed

with mutations in stem loop 1 (SL1, VLP) or deletion of stem loop 3 (ΔSL3). The ability of the SL1

or SL3 modifications to impact RNA packaging in NL4-3 and HIV 1086 were assessed using 293T

cell transient transfections and qRT-PCR with a gag primer set (**A and B**). The mean RNA copes/ml (+/-SEM) are shown. Viral particles (VP) lacking modifications to the RNA packaging sequence and media were used as positive and negative control respectively. Reverse transcriptase activity in the NL4-3 and HIV 1086 VP and VLP formulations were assessed using radioactive reverse transcriptase assay and densitometry readings on radiographical film (**C** and **D**). The ratio of RNA to reverse transcriptase activity was calculated for the SL1 VLP and ΔSL3 modified NL4-3 and HIV 1086 formulations (**E** and **F**).
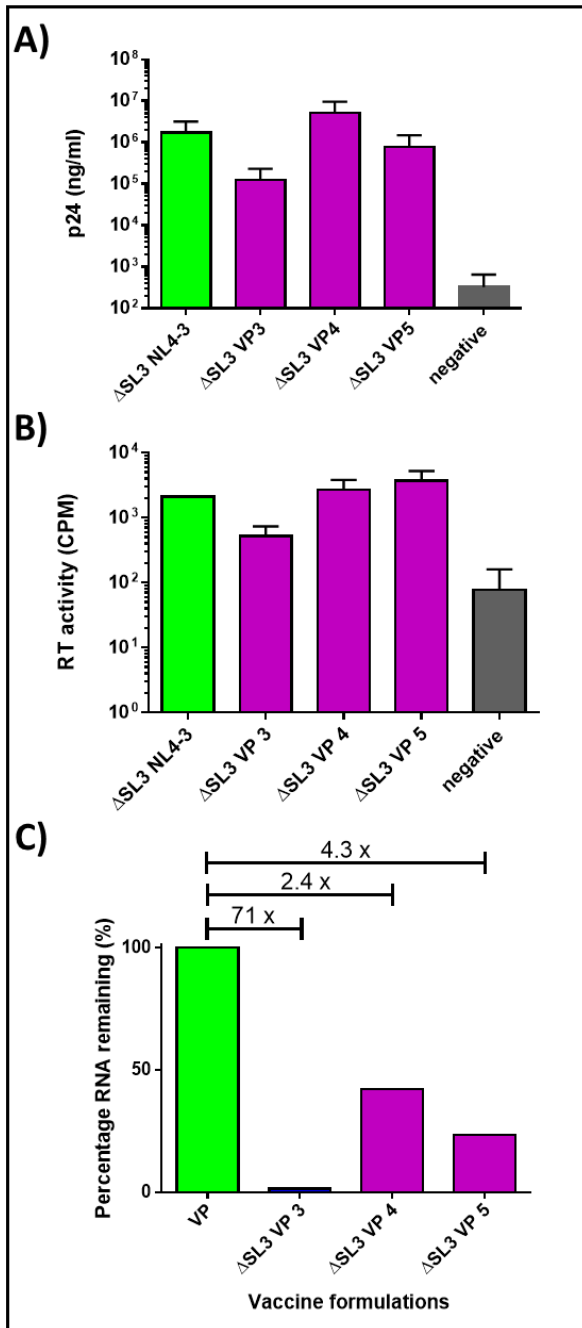
Figure 2-8S: The deletion of stem loop 3 (dSL3) causes RNA packaging defects in viral particles.

The ΔSL3 mutagenic primer was used in the nested PCR reaction for the five cDNA's derived from HIV+ volunteer samples. Of the five ΔSL3 VPs, only ΔSL3 VP 3, 4 and 5 resulted in successful recombinants in yeast. The DNAs were then used to transfect bacteria and subsequently transfect 293T cells. The purified ΔSL3 VP 3-5 were assayed for p24 content (**A**) in culture

supernatants by ELISA and for reverse transcriptase activity using a radioactive RT assay (**B**). The percentage RNA in ΔSL3 VP 3-5 were determined, by first isolating viral RNA and generating cDNA, before using qRT-PCR and a gag primer set to quantify viral genomic levels (**C**). Results for ΔSL3 VP 3-5 RNA levels are shown as percentage RNAs relative to the volunteer matched and unmodified VPs.
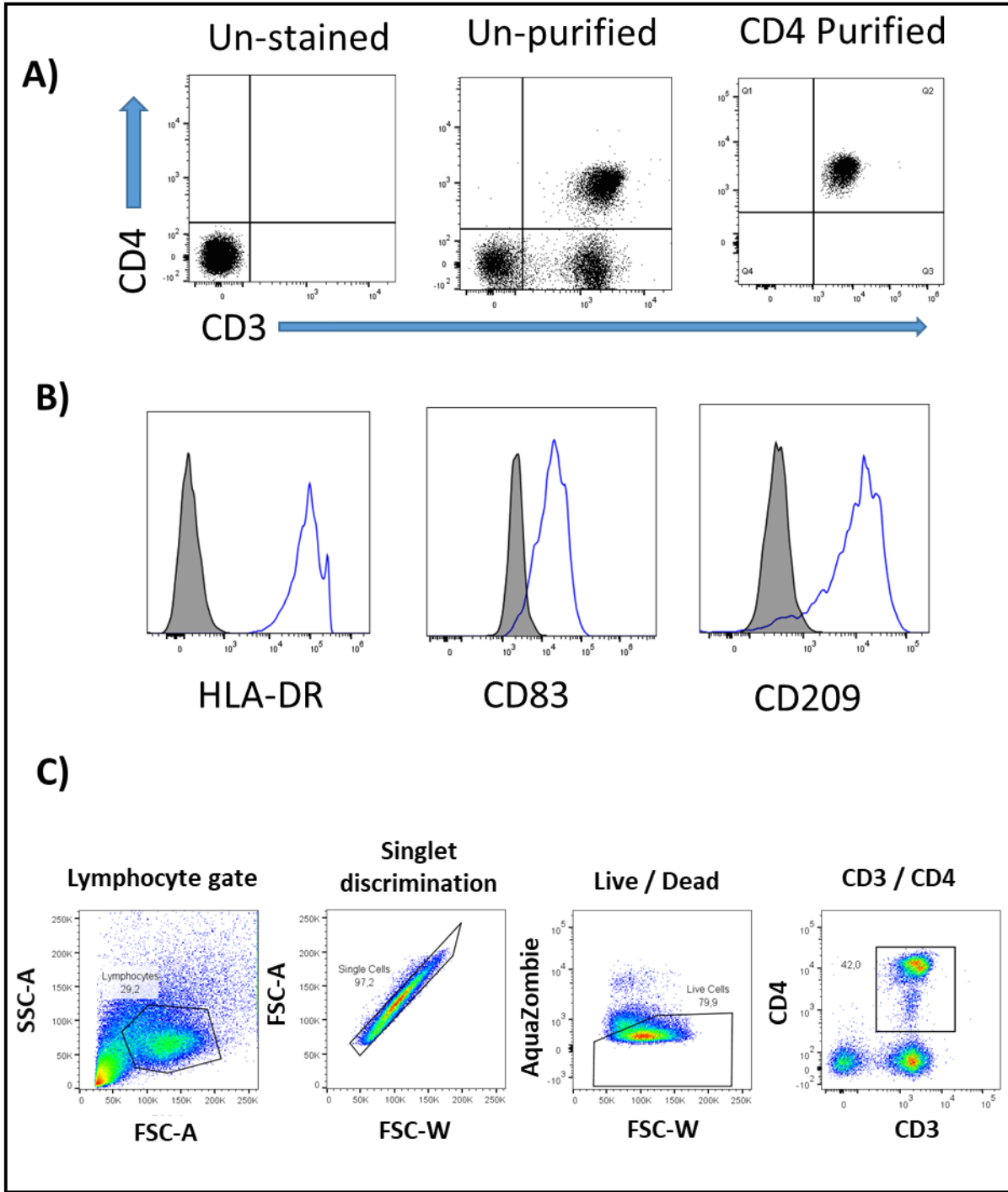
Figure 2-9S: Characterization of various PBMC cellular subsets.

Cryopreserved PBMC from fully consented HIV naïve volunteers under continuous anti-retroviral treatment where used in these studies. PBMC were defrosted and CD4+ T cells isolated by magnetic isolation and negative selection. The purity of the isolated CD4+ T cells was determined

by live/dead cell gating prior to CD3$^+$ and CD4$^+$ gating. A representative flow cytometry plot is shown (**A**). PBMC were defrosted to generate MDDC. Initially, PBMC were allowed to plastic adhere in tissue culture flasks monocytes before incubation for 6 days in the presence of IL-4 and GM-CSF. Immature DC were then phenotypically characterized by flow cytometry using HLA-DR, CD83 and CD209 antibodies (**B**). To evaluate Het_B_ACT-VEC and VP immunogenicity, the formulations were used to pulse the prepared MDDC overnight, followed by co-incubating the MDDC with autologous CD4$^+$ T cells. The flow cytometry gating strategy used to characterize define the CD4$^+$ T cells used in the immunogenicity studies is shown (**C**).

2.9 References

1.   Lederman, M. M. *et al.* A Cure for HIV Infection: "Not in My Lifetime" or "Just Around the Corner"? *Pathog. Immun.* (2016). doi:10.20411/pai.v1i1.133

2.   Rerks-Ngarm, S. *et al.* Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N. Engl. J. Med.* (2009). doi:10.1056/NEJMoa0908492

3.   Cohen, K. W. & Frahm, N. Current views on the potential for development of a HIV vaccine. *Expert Opinion on Biological Therapy* (2017). doi:10.1080/14712598.2017.1282457

4.   Stephenson, K. E., D'Couto, H. T. & Barouch, D. H. New concepts in HIV-1 vaccine development. *Current Opinion in Immunology* (2016). doi:10.1016/j.coi.2016.05.011

5.   Santra, S. *et al.* Breadth of cellular and humoral immune responses elicited in rhesus monkeys by multi-valent mosaic and consensus immunogens. *Virology* (2012). doi:10.1016/j.virol.2012.03.012

6.   Wang, S. *et al.* Polyvalent HIV-1 Env vaccine formulations delivered by the DNA priming plus protein boosting approach are effective in generating neutralizing antibodies against primary human immunodeficiency virus type 1 isolates from subtypes A, B, C, D and E. *Virology* (2006). doi:10.1016/j.virol.2006.02.032

7.   Pal, R. *et al.* Polyvalent DNA prime and envelope protein boost HIV-1 vaccine elicits humoral and cellular responses and controls plasma viremia in rhesus macaques following rectal challenge with an R5 SHIV isolate. *J. Med. Primatol.* (2005). doi:10.1111/j.1600-0684.2005.00120.x

8.   Lu, S., M. Grimes Serrano, J. & Wang, S. Polyvalent AIDS Vaccines. *Curr. HIV Res.* (2011). doi:10.2174/157016210794088290

9.   Bowles, E. J. *et al.* Comparison of neutralizing antibody responses elicited from highly diverse polyvalent heterotrimeric HIV-1 gp140 cocktail immunogens versus a monovalent counterpart in rhesus macaques. *PLoS One* (2014). doi:10.1371/journal.pone.0114709

10.   Kwong, P. D., Mascola, J. R. & Nabel, G. J. Broadly neutralizing antibodies and the search for an HIV-1 vaccine: The end of the beginning. *Nature Reviews Immunology* (2013). doi:10.1038/nri3516

11.   Fischer, W. *et al.* Polyvalent vaccines for optimal coverage of potential T-cell epitopes in global HIV-1 variants. *Nat. Med.* (2007). doi:10.1038/nm1461

12.   Gohain, N. *et al.* Molecular basis for epitope recognition by non-neutralizing anti-gp41 antibody F240. *Sci. Rep.* (2016). doi:10.1038/srep36685

13.   Hansen, S. G. *et al.* Immune clearance of highly pathogenic SIV infection. *Nature* (2013). doi:10.1038/nature12519

14.   Zhao, C., Ao, Z. & Yao, X. Current advances in virus-like particles as a vaccination

approach against HIV infection. *Vaccines* (2016). doi:10.3390/vaccines4010002

15. Pankrac, J., Klein, K. & Mann, J. F. S. Eradication of HIV-1 latent reservoirs through therapeutic vaccination. *AIDS Res. Ther.* **14**, (2017).

16. Moore, D. M., Arts, E. J., Gao, Y. & Marozsan, A. J. A yeast recombination-based cloning system to produce chimeric HIV-1 viruses and express HIV-1 genes. *Methods Mol. Biol.* (2005).

17. Dudley, D. M. *et al.* A novel yeast-based recombination method to clone and propagate diverse HIV-1 isolates. *Biotechniques* (2009). doi:10.2144/000113119

18. Marozsan, A. J. & Arts, E. J. Development of a yeast-based recombination cloning/system for the analysis of gene products from diverse human immunodeficiency virus type 1 isolates. *J. Virol. Methods* **111**, 111–120 (2003).

19. Weber, J. *et al.* Sensitive cell-based assay for determination of human immunodeficiency virus type 1 coreceptor tropism. *J. Clin. Microbiol.* (2013). doi:10.1128/JCM.00092-13

20. Hammonds, J., Wang, J. J., Yi, H. & Spearman, P. Immunoelectron microscopic evidence for tetherin/BST2 as the physical bridge between HIV-1 virions and the plasma membrane. *PLoS Pathog.* (2010). doi:10.1371/journal.ppat.1000749

21. Iwabu, Y. *et al.* HIV-1 accessory protein Vpu internalizes cell-surface BST-2/tetherin through transmembrane interactions leading to lysosomes. *J. Biol. Chem.* (2009). doi:10.1074/jbc.M109.058305

22. Archer, J. *et al.* Use of Four Next-Generation Sequencing Platforms to Determine HIV-1 Coreceptor Tropism. *PLoS One* (2012). doi:10.1371/journal.pone.0049602

23. Clever, J. L. & Parslow, T. G. Mutant human immunodeficiency virus type 1 genomes with defects in RNA dimerization or encapsidation. *J. Virol.* **71**, 3407–14 (1997).

24. Venner, C. M. *et al.* Infecting HIV-1 Subtype Predicts Disease Progression in Women of Sub-Saharan Africa. *EBioMedicine* (2016). doi:10.1016/j.ebiom.2016.10.014

25. Choi, E. *et al.* First Phase I human clinical trial of a killed whole-HIV-1 vaccine: Demonstration of its safety and enhancement of anti-HIV antibody responses. *Retrovirology* (2016). doi:10.1186/s12977-016-0317-2

26. Chakrabarti, L. A. *et al.* A Truncated Form of Nef Selected during Pathogenic Reversion of Simian Immunodeficiency Virus SIVmac239 nef Increases Viral Replication. *J. Virol.* (2003). doi:10.1128/jvi.77.2.1245-1256.2003

27. Learmont, J. C. *et al.* Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1. A report from the Sydney Blood Bank cohort. *N. Engl. J. Med.* (1999). doi:10.1056/NEJM199906033402203

28. Deeks, S. G. HIV: Shock and kill. *Nature* (2012). doi:10.1038/487439a

29. Arakelyan, A. *et al.* Flow virometry analysis of envelope glycoprotein conformations on

individual HIV virions. *Sci. Rep.* (2017). doi:10.1038/s41598-017-00935-w

30.   Lewis, G. K. The first 24 h: Targeting the window of opportunity for antibody-mediated protection against HIV-1 transmission. *Current Opinion in HIV and AIDS* (2016). doi:10.1097/COH.0000000000000319

31.   Deng, K. *et al.* Broad CTL response is required to clear latent HIV-1 due to dominance of escape mutations. *Nature* (2015). doi:10.1038/nature14053

32.   Schiffner, T., Sattentau, Q. J. & Dorrell, L. Development of prophylactic vaccines against HIV-1. *Retrovirology* (2013). doi:10.1186/1742-4690-10-72

33.   Nankya, I. L. *et al.* Defining the fitness of HIV-1 isolates with dual/mixed co-receptor usage. *AIDS Res. Ther.* (2015). doi:10.1186/s12981-015-0066-7

34.   Edgar, R. C. MUSCLE: A multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* (2004). doi:10.1186/1471-2105-5-113

35.   Edgar, R. C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* (2004). doi:10.1093/nar/gkh340

36.   Gouy, M., Guindon, S. & Gascuel, O. Sea view version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol. Biol. Evol.* (2010). doi:10.1093/molbev/msp259

37.   Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* (2013). doi:10.1093/molbev/mst197

Chapter 3

Title: <u>A targeted reactivation of latent HIV-1 using an activator vector.</u>

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3.1 Research in Context

Evidence before this study: The HIV-1 latent reservoir becomes established early after primary infection and remains despite continuous cART administration. Numerous approaches have sought to induce transcriptional activity of latent provirus, including: HDAC inhibitors, TLR agonists, PCK agonists, BET bromodomain inhibitors, etc. These approaches have had variable degrees of success in vitro but are largely ineffective at either the clinical or pre-clinical stages of development. Historic and emerging studies suggest that i) HIV-1 latency reversal can be achieved via TCR engagement, ii) latency reversal can be achieved using HIV-derived peptides, and iii) latent provirus has a high concentration within the HIV-specific T cell population. The effect of a polyvalent stimulator capable of *specifically* targeting the predominant reservoir is largely unknown.

Added value of this study: Unlike other latency reversing agents to-date, ACT-VEC is a polyvalent formulation that can specifically target HIV-specific CD4+ T cells for transcriptional reactivation. Our study identifies a novel immunotherapeutic strategy that is at least an order of magnitude more potent than other drugs currently under clinical development. Additionally, we show that the inducible HIV-1 proviral reservoir is enriched within HIV-specific CD4+ T cells. Our study further elucidates several mechanistic steps that contribute to an optimized latency reversing response via antigen presentation.

Implications of all the available evidence: The ability of HIV-1 to establish latent provirus within the CD4+ T cell reservoir is a major reason for virus recrudescence upon cessation of cART. Identifying novel mechanisms to deplete this reservoir are of great research priority, as current methods fail to effectively target latently infected cells for reactivation and eradication. Importantly, our work shows that the proviral reservoir established during acute infection is non-random and enriched for TCRs specific to HIV antigens. Furthermore, we are the first to describe a method to induce HIV latency reversal by targeting HIV-1 specific T cells using an immunotherapeutic approach.

3.2 Introduction

Despite the success of combination antiretroviral therapy (cART) at reducing HIV viral loads to undetectable levels, cART remains non-curative[1]. During HIV infection, rare integration events of replication-competent virus occurs, and for reasons not fully understood, remain as transcriptionally silent provirus within regions of host DNA. The resulting viral reservoir is transiently non-replicating, temporarily hidden from immune surveillance and not susceptible to the protective actions of cART[2–5]. Following cART initiation, latent HIV undergoes a multiphasic decay, with the majority of provirus being present in long lived memory CD4 T cells[6,7]. Upon cART cessation, rapid viral recrudessence and resumption of disease occurs.

Many curative approaches during cART have been centred on "Shock and Kill", where latency reversal agents (LRAs) reactivate transcription and translation of the otherwise latent provirus[8]. In theory an effective LRA therapy should induce de novo HIV-1 protein expression within previously latently infected cells, enabling their immune recognition and destruction, with any released virus neutralized by ongoing cART[9]. Histone Deacetylase Inhibitors (HDACi) are potential LRAs, based on their histone hyperacetylation, chromatin remodeling and pleotropic induction of gene expression. Potency of the HDACi shock can be significantly enhanced when combined with protein Kinase C (PKC) agonists[10]. However, high toxicity/low therapeutic index of most PKC agonists and HDACi, combined with their negative immuno-modulatory effects and lack of specificity has raised questions on their utility[11–13]. The modest effects of these LRAs to impact the reservoir size in clinical studies has renewed the urgent need to develop novel, safe, efficacious and targeted LRA approaches.

CD4 T cell populations are the major proviral reservoir, but only a fraction of these cells harbors inducible, replication competent virus (0.0001%). In fact, more recent studies have suggested that a mere 2.4% of proviruses are intact with the reminder harboring fatal mutations and deletions[14]. Prior studies have suggested that latent HIV may be concentrated in CD4 T cells bearing T cell receptors (TCRs) specific for HIV antigens[15,16]. During acute/early infection, HIV drives clonal expansion of HIV-specific T cells, thereby providing an abundance of susceptible target cells to fuel exponential increase in HIV load[17]. By extension, in patients starting a cART

regimen at acute/early infection, the latent HIV may be housed in the HIV-specific memory CD4 T cells. Interestingly, despite evidence suggesting the latent reservoir is established early after infection, data now supports that within infected individuals, the virus that was replicating immeditaley prior to suppressive cART may predominate within the HIV-1 DNA reservoir[18,19]. We therefore asked the question, could an immunotherapy based on a heterogeneous, polyvalent, HIV-1 vaccine consisting of HIV virus-like-particles (VLPs) (termed activating vector, ACT-VEC), serve as a highly specific and effective LRA "shock"? In this scenario, ACT-VEC would be presented to HIV-specific memory CD4 T cells, with signaling through the TCR activating transcription of the otherwise latent HIV-1 provirus, ultimately leading to expression of viral proteins and possible release of HIV. To address this question, we recruited HIV+ volunteers that were diagnosed and cART treated during acute/early infection (AC-cART). Purified CD4 T cells from these volunteers (AC-cART CD4 T cells) were either presented with a heterologous ACT-VEC VLP formulation or other non-HIV recall antigens by autologous monocyte-derived dendritic cells (MDDCs). We measured T cell stimulation and any HIV RNA production due to latency reversal. PMA/Iono, Bryostatin, various HDACi and non-HIV viral particles were used as LRA controls. These results show that ACT-VEC is a promising immunotherapeutic LRA for the induction of replication competent HIV-1 from latently infected CD4 T cells and that the inducible reservoir may indeed be enriched within HIV-specific CD4 T cells.

3.3 Results

3.3.1 HIV+ volunteer characteristics

The primary objective was to determine if heterologous ACT-VEC VLPs are an effective LRA for CD4 T cells derived from HIV+ volunteers (N=9, mean age of 38), diagnosed and receiving cART at acute/early stage of infection (AC-cART patients) (Table 3-1). The average time between the estimated date of HIV seroconversion (EDS) and diagnosis was 69 days (range = 6.5 – 207.5 days) while the time between HIV diagnosis and first line cART initiation was 32 days (range = 18-54 days). During cART treatment, volunteers rapidly suppressed viral loads to <50 copies/mL and maintained suppression throughout the study (Figure 3-1). Average duration of cART treatment (with undetectable viremia) prior to PBMC sampling for this study was 884 days (range = 618-1206 days). At the time of PBMC sample collection, CD4 counts ranged from 456 – 1179 cells/μl

(mean = 772 cells/µl). All volunteers were Hepatitis B and C negative at enrollment (Table 3-1). The mean frequency of HIV DNA$^+$ cells, representing the full spectrum of the proviral reservoir (i.e. replication competent, replication defective and graveyard viruses) was determined to be 1/4133 CD4 T cells by qRT-PCR.
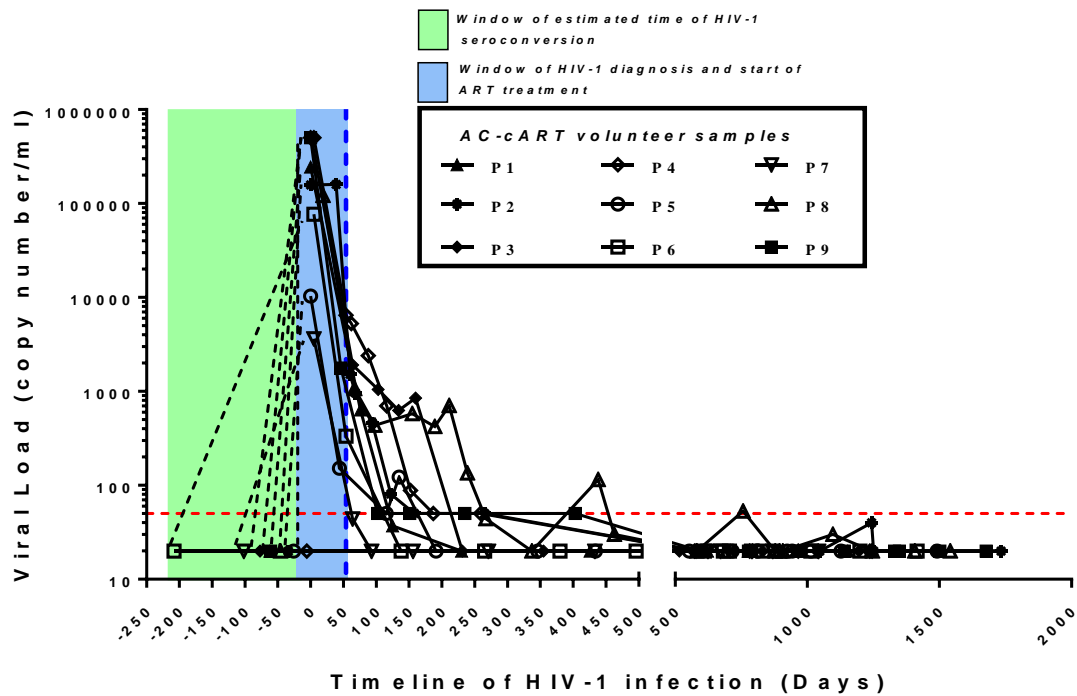
Figure 3-1: Figure 1. All nine HIV volunteers enrolled in this study durably suppressed viremia upon cART initiation.

A total of nine HIV infected individuals that were diagnosed and treated during acute/early stage of HIV infection were enrolled into this study. Upon diagnosis and initiation of cART, the treated HIV+ volunteers durably suppressed viral replication to <50 HIV copies/mL (---). All volunteers were fully consented to provide a large blood draw (300-500mL) for PBMC isolation and use in the latency reversal studies. The estimated date/time of seroconversion (EDS) was calculated as the midpoint between the most recent negative and the first positive test for the volunteers and is shown by the green shaded window ( ). The time window in which HIV diagnosis and cART treatment occurred is shown by the blue shaded area ( ).

3.3.2 ACT-VEC can cause HIV-1 latency reversal in CD4 T cells.

During acute HIV infection, the population of CD4 T cells with TCRs specific to viral antigens expands significantly. These activated CD4 T cells either: 1) provide help for anti-viral immune responses and eventually undergo caspase 3-mediated apoptosis, 2) revert to a resting memory phenotype and avoid apoptosis, 3) become productively HIV-infected and die via viral cytopathic effects (vCPE) or cytotoxic responses or 4) become latently infected during the transition into a memory phenotype[20–25]. Based on these cellular fates and because HIV preferentially infects activated CD4 T cells, we propose that initiation of cART during acute/early infection may trap latent HIV within the HIV-specific CD4 T cell population. Therefore, cellular reactivation via the TCR and associated co-stimulatory molecules should lead to the initiation of multiple intracellular signaling cascades and increased expression of a plethora of cellular transcription factors, which may in turn, lead to mRNA transcription from the HIV proviral DNA[26].

The heterologous ACT-VEC LRAs used in this study are VLPs derived from the quasi-species of patients infected with subtype B HIV-1[27]. ACT-VEC is morphologically indistinguishable from wild type virus, containing all viral proteins, but is devoid of HIV RNA[27]. Monocyte derived dendritic cells (MDDC), derived from the PBMCs of AC-cART patients, were antigen-loaded with ACT-VEC (ACT-VEC+MDDC) overnight before washing the MDDC and then co-culturing with the autologous purified AC-cART CD4 T cells for three days (Figure 3-8S). When ACT-VEC+MDDC were co-cultured with AC-cART CD4 T cells, HIV genomic RNA was readily detectable in culture supernatants from each of the nine HIV+ donor samples (Figure 3-2, A and B), representing an average 7.1-fold increase in HIV RNA compared to that from T cells incubated with unpulsed MDDC (Figure 3-2G). To ensure *de novo* HIV production was from only latently infected cells and not due to HIV propagation, HIV entry inhibitor, Enfuvirtide, was added (20 μM) to all samples to block new HIV infections. A 6.8 and 4.98-fold increase in HIV RNA was released from AC-cART CD4 T cells into supernatant when they were treated with ACT-VEC+MDDC than when treated with polyclonal T cell activator PMA/Iono, or when MDDC were antigen loaded with a cocktail of CMV peptide, Tetanus Toxoid, and Flu M1 (CTF) (Figure 3-2G). To exclude the possibility that ACT-VEC-associated increases in HIV RNA resulted from heightened levels of MDDC maturation, we measured the surface maturation markers CD40, CD80, and CD83 before and after stimulation
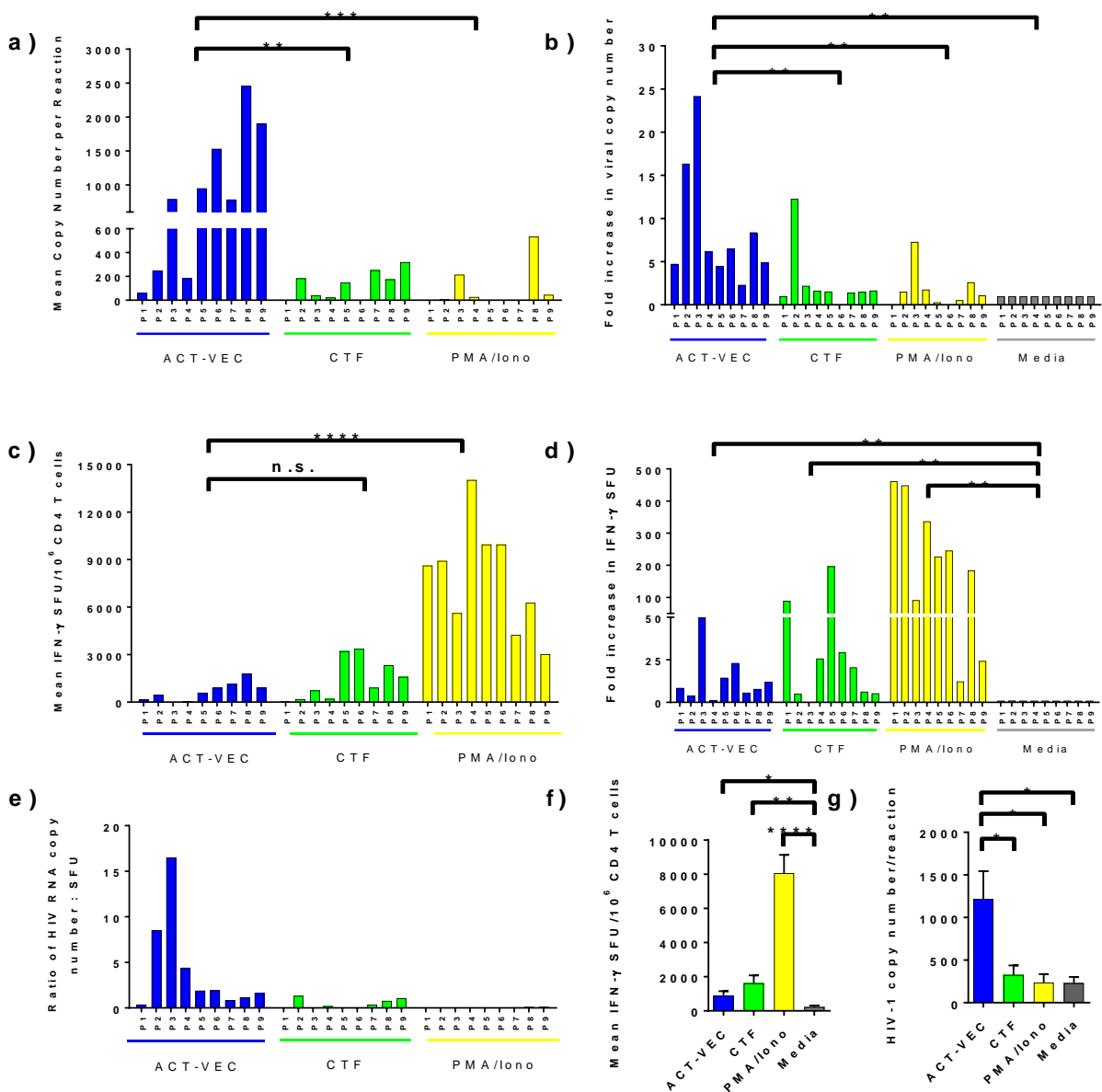
Figure 3-2: ACT-VEC induces potent latency reversal within patient-derived CD4 T cells.

cART treated HIV+ volunteers (*n*=9) with durable viral suppression (<50 HIV copies/mL were

enrolled in this study. ACT-VEC (5ug / mL) or a cocktail of Flu M1, Tetanus toxoid, CMV peptide

pool (CTF) were used as HIV-specific or non-specific stimuli to pulse volunteer MDDCs. The pulsed

MDDC were then co-incubated with autologous CD4 T cells. PMA/Iono and media served as assay

positive and negative controls, respectively. **(a)** After 3 days of co-culture, mean viral copy number was determined via qRT-PCR from culture supernatants. **(b)** Copy numbers derived from culture supernatants were represented as fold increase over the media control. **(c)** Cells stimulated with ACT-VEC, CTF and PMA/Iono were cultured overnight in an IFN-γ ELISpot to determine the CD4 T cell antigenicity. **(d)** Volunteer-specific IFN-γ spot forming units (SFUs) were counted /10^6 CD4 T cells, and fold increase calculated over the media control (±SEM). Statistical significance was calculated using a Wilcoxon matched pairs signed rank test. **(e)** The relationship between CD4 T cell activation and the quantity of induced HIV-1 virus is shown. Grouped samples were used to determine the mean effect (±SEM) of the stimulations on antigenicity and induced copy number (**f and g**) with statistical significance ascertained by Mann-Whitney unpaired t-test.

via flow cytometry. Maturation induction in response to ACT-VEC particles was compared to both i) pre-processed peptide, in the form of Env/Gag/Pol pooled peptide and ii) a mixed peptide/protein formulation of CTF. Compared to CTF, ACT-VEC expressed similar levels of MDDC maturation across all three markers, indicating that any increased detection of HIV RNA in these studies were not, due to drastically differing levels of maturation of the MDDC (Figure 3-9S, A and B). To determine the potential of contaminating CD4+ T cells within the plastic-adhered MDDC population, flow cytometry was routinely performed using HLA-DR, CD80, CD83, CD40 and CD11c. In all instances, the purity of the MDDC population 6-days after plastic adherence was between 95-99%. As additional non-HIV based latency reversal controls, and to help demonstrate the antigen-specificity of the observed latency reversal associated with ACT-VEC VLP, SIVmac251 and FIV viral particles were made and both failed to induce detectable amounts of HIV-1 RNA (>30 copies/reaction) within culture supernatants (Figure 3-11S G). This further suggests that only HIV and no other related lentiviruses presented by MDDC, can trigger HIV latency reversal in AC-cART CD4 T cells.

It is important to note HIV-1 RNA was measured in supernatant with a qRT-PCR assay targeting the gRNA 5'LTR region (R-U5) found only within inducible wild type HIV-1 but absent in genomeless ACT-VEC VLPs[27]. In support of this detection specificity, no induced HIV-1 or contaminating ACT-VEC was detected in any HIV⁻ donor control experiments using this qRT-PCR assay (Figure 3-10S, A and E). As a further verification that the HIV-1 RNA detected by qRT-PCR was not derived from ACT-VEC, we RT-PCR amplified and then sequenced the HIV-1 5'LTR region derived from supernatant following latency reversal with the CD4 T cells. Illumina MiSeq confirmed the presence of HIV-1 5'LTR sequences and also the absence of our previously published mutations introduced into the DNA vector used to generate ACT-VEC VLP[27]. In the AC-cART volunteers, only a single HIV-1 sequence was identified/volunteer by sequencing which is consistent with the high levels of conservation of the 5'LTR and low levels of diversity of HIV provirus within acute HIV infections[28].

We suspect that the ability of ACT-VEC to induce latency reversal relates to the specific activation of memory CD4 T cells with TCRs specific to HIV as an antigen. However, HIV-specific memory CD4 T cells may only comprise a small proportion of all memory T cells within infected

individuals despite large numbers of these cells being generated during early infection[15]. Our adult participants likely encountered flu, CMV and/or tetanus (as well as other antigens) multiple times during their life through vaccination, infection or environmental exposure. Based on these assumptions and the fact that standard vaccines such as Influenza, Hepatitis B, Pneumococcus and oral cholera vaccine have been described to cause transient elevations in HIV expression within individuals on suppressive antiretroviral treatment, we measured the number of AC-cART CD4 T cells that were responsive to ACT-VEC as well as to common recall antigens, such as CTF[29–34]. Additionally, cells were stimulated with the T cell mitogen, PMA/Iono, to determine the effect of a potent, non-specific activation on latency reversal. We utilized PMA/Iono as our assay positive control over other strong T cell mitogens such as αCD3/CD28, and PHA-L, based on our preliminary findings using flow cytometry (Figure 3-9S, C to E) and IFN-γ ELISpot based protocols (Figure 3-9S, F and G). In these initial experiments, the culminative result demonstrated that PMA/Iono was the most potent control stimulation, followed by aCD3/CD28 and PHA-L. This was corroborated by findings from other research groups showing PMA/Iono has accelerated latency reversing kinetics when compared to the other stimulations, while reaching a similar level of peak activation[35,36]. As shown, ACT-VEC+MDDC was clearly antigenic when presented to the CD4 T cells with generation of approximately 4-fold more CD4 T cell spot forming units (SFU)/10^6 cells relative to the untreated controls (average of 891 versus 222 SFU/10^6, **$p<$

VP0.0296) (Figure 3-2, C and D). Stimulation of T cells with CTF+MDDC (**$p=0.0012$) and PMA/Iono (****$p=0.0001$) resulted in 7.31 and 36.4-fold increases in IFN-γ SFU relative to the untreated controls (Figure 3-2F) and were stronger stimuli than ACT-VEC+MDDC (1.8 and 9.1-fold increase in SFUs, respectively).

We next measured the ratio of HIV-1 RNA production versus CD4 T cell activation (Figure 3-2E and Figure 3-10S, C). If latent HIV is housed primarily in HIV-specific CD4 T cells, the virus released into supernatant per activated CD4 T cell should be greatest under the MDDC+ACT-VEC conditioning, which was indeed the case (Figure 3-2G). The numbers of ACT-VEC activated CD4 T

cells (based on SFU/10^6) correlated with the induced HIV copy number (**p=0.0045) (Figure 3-11S, A and B). Significantly less HIV-1 RNA in culture supernatant was observed per activated CD4 T cell when treated with either unpulsed MDDC, CTF+MDDC, and even PMA/Iono controls (Figure 3-2G). As a non-specific CD4 T cell activator, the mitogen PMA/Iono stimulates substantially more cells that either ACT-VEC or CTF and yet, less HIV is released from reactivated latent pool in these PMA/Iono activated T cells. This finding coupled with the inability of CTF to trigger significant HIV latency reversal supports our hypothesis that a sizable reservoir of latent HIV is housed primarily in HIV-specific CD4 T cells of volunteers treated with cART at acute/early infection. As expected, no HIV-specific CD4 T cell activation or induced HIV genome was detectable in CD4 T cells of HIV$^-$ donors cocultured with autologous MDDC pulsed with ACT-VEC (Figure 3-10S, A and E).

3.3.3 ACT-VEC mediated latency reversal induces replication competent virus.

Within HIV infected individuals receiving cART, proviral DNA integrated into host DNA may be inert or can be induced to produce replication competent as well as defective and dead HIV. While reactivation and elimination of all inducible, replication competent provirus will be necessary for a sterilizing cure, any reductions in proviral reservoir might significantly reduce the length of time individuals may be required to take cART for the chances of viral rebound to be diminished. To evaluate whether HIV antigens delivered by ACT-VEC induces replication competent HIV from activated CD4 T cells, we monitored HIV propagation following ACT-VEC stimulation (in the absence of T-20; enfurvitide (fusion inhibitor)) in an assay where MDDC+ACT-VEC/CD4 T cells were separated by a 0.4µm membrane from the permissive MOLT4 CCR5 cells (Figure 3-3A). In this assay, HIV produced from activated T cells in the apical chamber can flow through, infect and propagate in the susceptible MOLT4 CCR5 cells in the basolateral chamber. By removing the apical chamber, we can accurately quantify the induced and replication competent virus in the basolateral chamber without a continuous influx of virus from the apical chamber confounding the results. Furthermore, the format of this viral outgrowth experiment enabled us to propagate virus indeterminately, using a low number of stimulated cells (1x10^5 T cells) and without the need for sub-culturing. Culture supernatants were collected every 3 days and assessed for viral RNA using qRT-PCR (Figure 3-3B). If replication competent virus was induced by the stimuli and subsequently infected the MOLT4 CCR5 cells within the basolateral

chamber, we would expect a logarithmic increase in the amount of viral RNA in culture supernatants between early and later time points. In this outgrowth format, we detected a pronounced increase in HIV-1 RNA via qRT-PCR by day 6, while day 3 was undetectable. This represents 2-3 log amplification of virus. After day 6, there was a marked reduction in detectable viral RNA (Figure 3-3B). The detection of virus on day 6 compared to day 3 represented a slight delay when compared to the previous latency reversal ELISpot format (Figure 3-2), which can be explained by the greater volume of culture supernatant involved in the assay and the fact that the induced virus is infecting the MOLT4 CCR5 cells. To further evaluate this finding, we conducted latency reversal experiments on samples from two volunteers under sub-culturing conditions (1/3 split) to determine if we could detect viral propagation (Figure 3-3C). As shown, we detected significantly increasing levels of HIV-1 p24 and viral RNA in culture supernatants over time (**p=0.0078), which culminated at days 9 and 12 (Figure 3-3, C and D). The increased production/propagation of HIV over time in this sub-culturing experiment suggests ACT-VEC induces infectious virus from the latent pool.
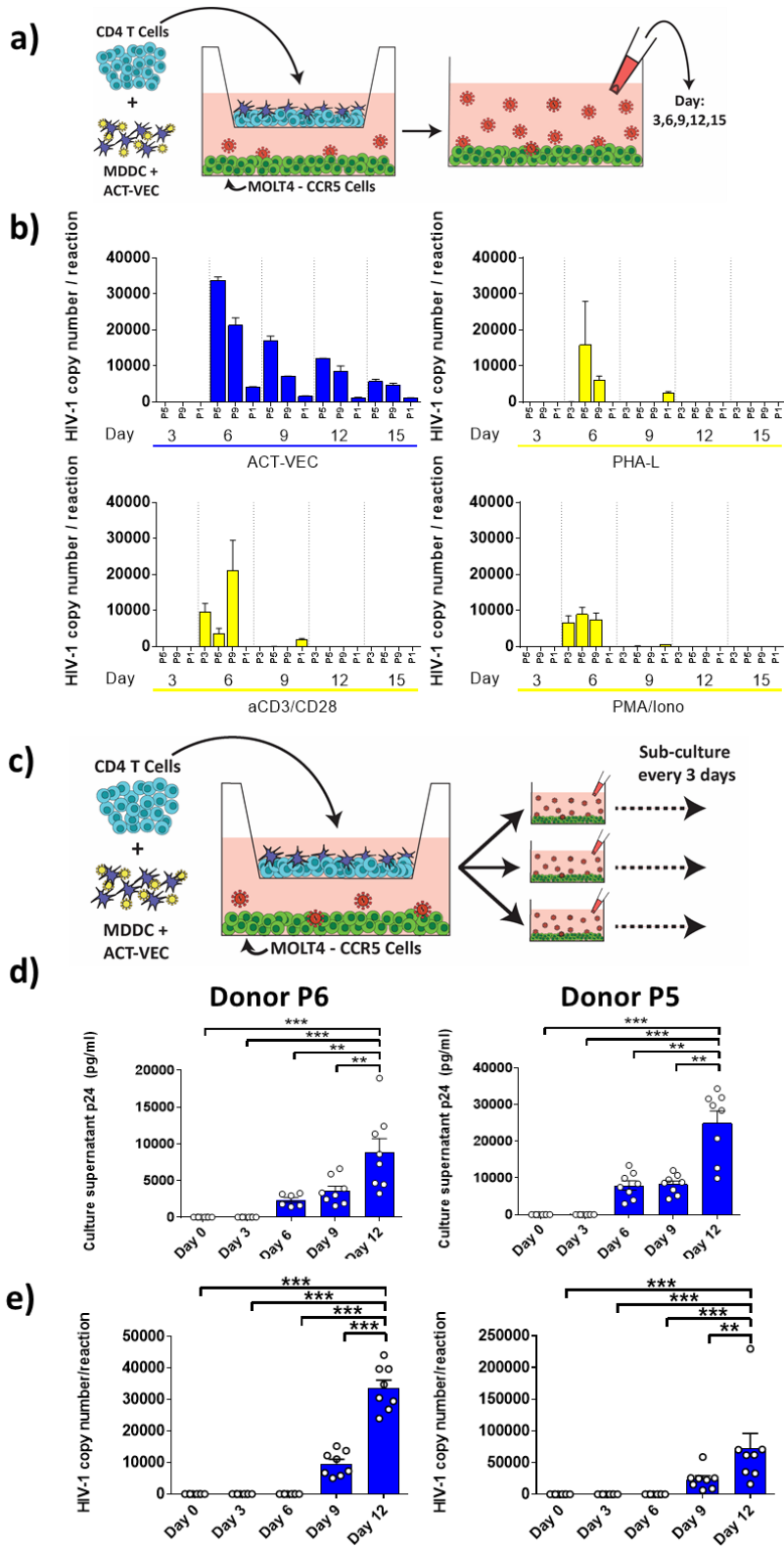
Figure 3-3: MDDC presentation of ACT-VEC to T cells induces replication competent virus though contact dependent mechanisms.

ACT-VEC pulsed MDDC were used to stimulate autologous CD4 T cells in Spin-X columns. **(a)** MOLT-4/CCR5 cells were added to the basolateral chamber of the column to propagate induced virus capable of replicating. After 3 days of stimulation and co-culture, the inserts containing MDDC-CD4 T cells were removed and MOLT-4 CCR5 cells were plated into a 24 well plate and cultured for a further 12 days. **(b)** MOLT-4 CCR5 cells from outgrowth experiments were cultured for 15 days and viral copy number was analyzed in the culture supernatants every three days (n=3). **(c)** A similar viral outgrowth assay was set up whereby after 3 days of MDDC-CD4 T cell co-culture, the MDDC-CD4 T cell insert was removed and the MOLT4/CCR5 cells were split evenly into three plates. Samples were subsequently sub-cultured at 3-day intervals. **(d)** The two donor (n=2) samples tha t were sub-cultured were maintained over time to observe if viral propagation would occur. Viral p24 was measured by ELISA every 3 days. Viral p24 results are shown for days 9 and 12 (+/- SEM) **(e)** Viral copy numbers in culture supernatants were also determined by qRT-PCR every 3 days. Results for qRT-PCR are shown for days 9 and 12 (+/-SEM). Statistical significance was ascertained by Mann-Whitney unpaired t-test.

Deep gene sequencing of the propagated virus (C2-V3 region of Env) following ACT-VEC stimulation revealed 2 distinct virus clusters (Figure 3-4, A and B). For each of two AC-cART patients, replicate ACT-VEC stimulations (x7) of a limited number of CD4 T cells resulted in the propagation of a population of related HIV clones as well as some HIV-1 clones unique to only specific replicates. Of note is the identification of cellular proviruses (Red) with genetic homology to the induced viruses (Blue) in both volunteer samples (Figure 3-4B). Within each latency reversal assay (LR1-7) and excluding for the possibility that latently infected CD4 T cell clones might be represented more than once in each well[37], if each unique virus was induced from one latently infected CD4 T cell, we can extrapolate the data and estimate the minimal ACT-VEC inducible reservoir to be ~14 cells/million. This is substantially greater than the 1 cell/million cited in earlier publications and less than the 60 cells/million that has been estimated in other studies[38–40].
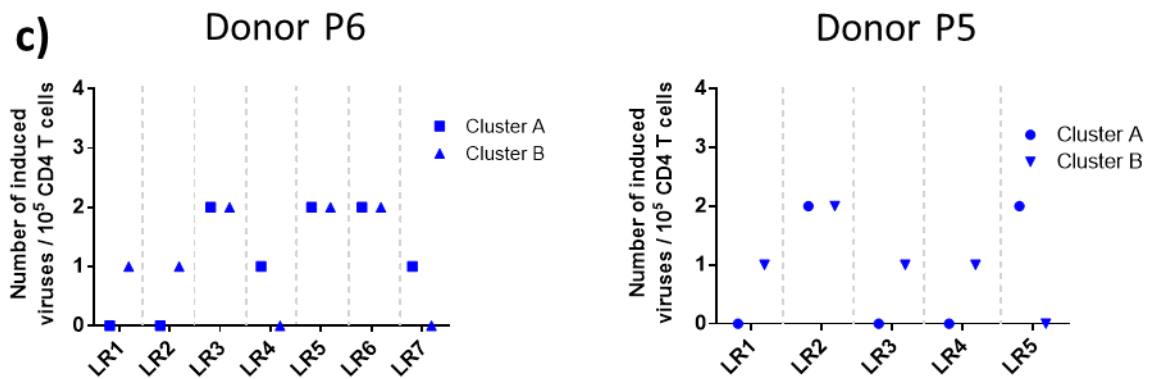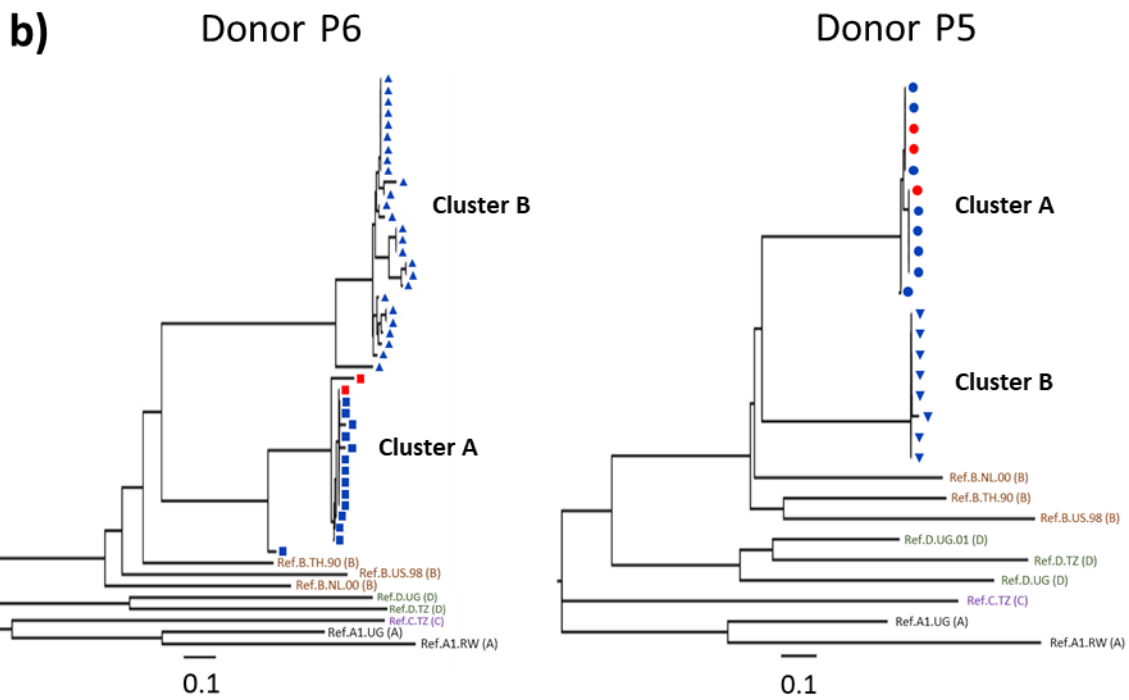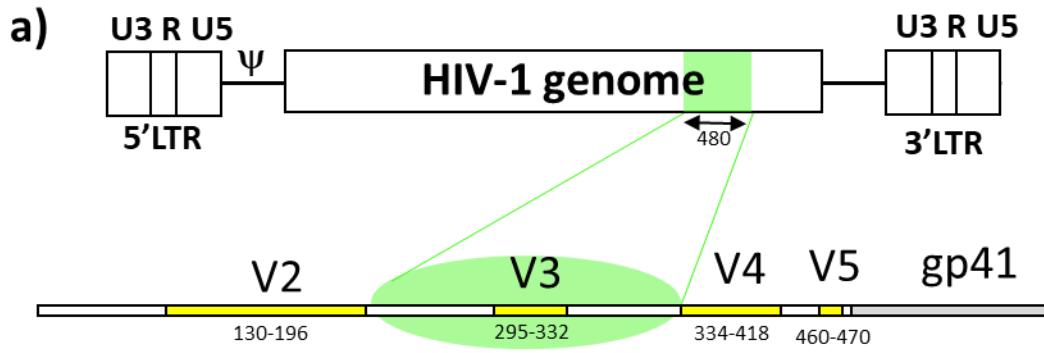
Figure 3-4: ACT-VEC-induced HIV-1 exhibits limited genetic diversity.

ACT-VEC pulsed MDDC were used to stimulate autologous CD4 T cells in Spin-X columns. MOLT-4/CCR5 cells were added to the basolateral chamber of the column to propagate induced virus capable of replicating. After 3 days of stimulation and co-culture, the apical insert containing the MDDC-CD4 T cells was removed and the MOLT-4 CCR5 cells were plated into a 24 well plate and cultured for a further 12 days. **(a)** Illumina MiSeq deep gene sequencing of the C2-V3 region of Env from ACT-VEC induced HIV-1 was carried out on day 12 culture supernatants. **(b)** Monophyletic viral clusters corresponding to the induced HIV-1 (Blue), were compared to HIV DNA from isolated provirus (Red) to demonstrate genetic homology. Reference sequences for subtypes A,B,C and D were added for comparison. **(c)** Latency reversal assays (LR1-7) were conducted multiple times with the induced virus sequenced to determine the number of times and variability that a particular viral sequence cold be identified within culture supernatants.

3.3.4 ACT-VEC is a more promising LRA compared to HDACi's and PKC agonist monotherapies.

ACT-VEC as a promising LRA was compared to the clinically relevant LRAs including Panobinostat, Vorinostat, Romidepsin and Bryostatin. (Figure 3-5). We initially tested the various HDACi and PKC agonists on the J-Lat 6.3 cell line to verify concentrations of drug that enable detectable latency reversal. As shown, the PKC agonist and HDACi alone or in combination, induced HIV-1 latency reversal in the J-Lat cell line (Figure 3-12S, F and G), consistent with previous studies. However, while we were able to detect latency reversal in the J-Lat cell line, we were unable to detect significant levels of HIV-1 RNA release from AC-cART CD4 T cells treated with HDACi's or Bryostatin monotherapies. While HDACi and PKC agonists were poor inducers of HIV in primary cells, ACT-VEC+MDDCs (*p= 0.0159) again showed significant latency reversal properties (Figure 3-5, A and B). Interestingly, ACT-VEC did not induce latency reversal in the J-Lat cell line, which suggests that, by itself, it is unable to induce latency reversal, probably because there is no MDDC-mediated presentation. Of note, Bryostatin in combination with Panobinostat (*p=0.031) or Romidepsin (**p=0.0079) was able to induce significant levels of detectable HIV-1 latency reversal, but this was less than observed with ACT-VEC. These findings demonstrate that ACT-VEC treatment of latently infected CD4 T cells is a stronger LRA than the tested HDACi and PKC agonists in primary human cells.

In these studies, ACT-VEC+MDDC was also found to be slightly more antigenic than the unstimulated control and individual HDACi's (Figure 3-5, C and D) as measured by IFN-γ ELISpot analyses. However, when comparing Bryostatin alone (*p<0.05) and the combination of Bryostatin with Romidepsin or Panobinostat, ACT-VEC was found to be less immune-stimulatory for AC-cART CD4 T cells. Again, when ACT-VEC latency reversal is evaluated based on the number of activated CD4 T cells, we demonstrate that ACT-VEC induces substantially more HIV-1 release on a per T cell basis than these HDACi or PKC agonists, suggesting ACT-VEC might be specifically targeting latency reversal in HIV-specific cells.
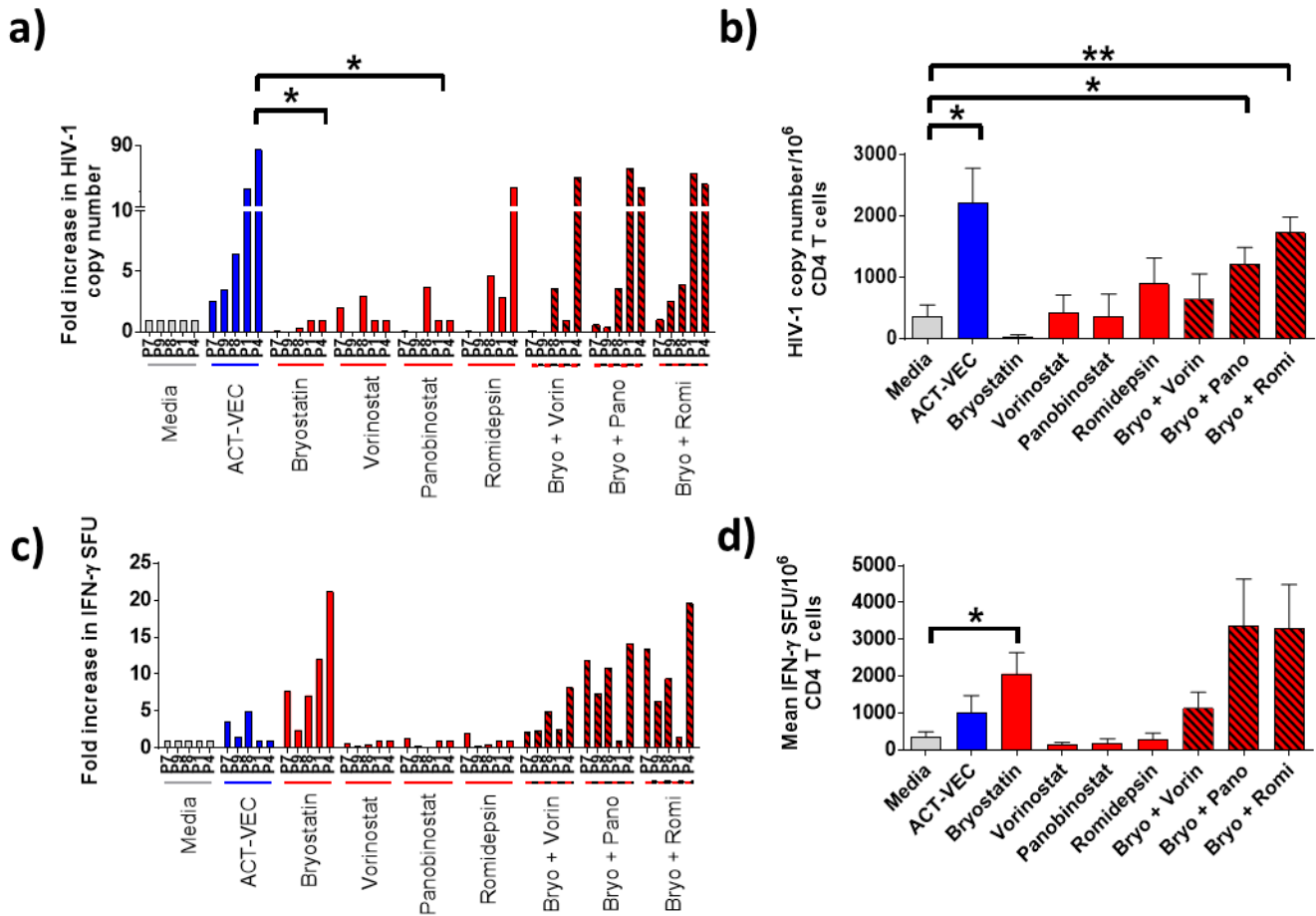
Figure 3-5: ACT-VEC is a more robust LRA than HDACi and PKC agonist regimens.

ACT-VEC+MDDC were used to stimulate autologous CD4 T cells from HIV infected donor cells (*n*=5). ACT-VEC stimulations were compared to Bryostatin, Vorinostat, Romidepsin and Panobinostat monotherapies as well as Bryostatin/Vorinostat, Bryostatin/Romidepsin and Bryostatin/Panobinostat combination therapies. (**a and b**) The induced viral copy number was determined after 3 days of cell culture in HIV donor samples by qRT-PCR. (**c and d**) To determine CD4 T cell activation, IFN-γ ELISpot was performed (100,000 cells/well) on HIV+ cells after overnight incubation. Results are shown as fold increase over media or group means of IFN-γ SFUs /10^6 CD4 T cells (±SEM). Statistical analysis was assessed by Mann-Whitney unpaired t-test (* $p<0.05$ and ** $p<0.005$).

3.3.5 ACT-VEC mediated HIV latency reversal is partially controlled by MDDC mediated contact with CD4 T cells and signaling through the Src family member Lck.

Understanding exactly how ACT-VEC functions as a latency reversal agent is now a priority, as is determining the fate of the cell reservoir upon ACT-VEC mediated latency reversal. Antigen processing, cell-cell contact, TCR-MHC II and co-stimulatory interactions, as well as soluble secreted factors are all likely to be important mediators involved in latency reversal. With that said, we noted that volunteer P7, whose CD4 T cells were consistently the weakest responders to ACT-VEC + MDDC (Figure 3-6A), was the only donor to be on a cART regimen involving a protease inhibitor (PI). The fact that PIs 1) have previously been shown to alter antigen processing and presentation, leading to changed frequencies and patterns of MHC displayed peptides and, 2) have reduced HIV reactivation potential, further suggests that antigen presentation and contact between MDDC and CD4 T cells may be important[41–43]. To confirm the role of MHC presentation on T cell activation and subsequent latency reversal, we performed our latency reversal assays in the presence of either the PI, Pepstatin A, or Chloroquine (CQ). Chloroquine is a lysosomotropic agent that prevents acidification of the endosome[44], and further prevents the fusion of the endosome and lysosome. This ultimately leads to insufficient peptide processing and poor presentation by MHC complexes. By limiting MHC II presentation of HIV derived peptides with either Pepstatin A or CQ, CD4 T cell activation was reduced by 72.9% and 96.2%, respectively, as compared to ACT-VEC stimulation in the absence of an inhibitor (Figure 3-6B). This marked reduction in T cell activation also had implications on latency reversal, wherein ACT-VEC+PI and ACT-VEC+CQ conditions both reduced HIV copy number by approximately 55% (Figure 3-6C). To build upon the previous result, we sought to determine the importance of MDDC - CD4 T cell contact at influencing HIV-1 latency reversal. Therefore, we co-cultured ACT-VEC+MDDC/CD4 T cells or separated the cells using a 0.4μm membrane. As expected, we show that MDDC-CD4 T cell contact is important for the high levels of latency reversal and viral budding seen in these studies (Figure 3-6, D and E). It is, however, difficult to sort out the master "regulator" of latency reversal by ACT-VEC, considering the multipronged signaling cascade from the TCR, co-stimulatory molecules and cytokine/chemokine receptors following this antigen presentation. This is further complicated by the diversity in proviral integration site, meaning

multiple signals are probably required to initiate latency reversal. Despite this, we evaluated the impact on ACT-VEC mediated CD4 T cell activation and latency reversal by using an inhibitor of T cell activation i.e. Dasatinib. The T cell mitogens used as controls in these latency reversal studies act through differing mechanisms. As shown in Figure 3-6F, and as reported by others[45], Dasatinib works through inhibition of Lck, which is a protein tyrosine kinase that acts proximal to the TCR. Hence, it is unsurprising that Dasatinib was ineffective at inhibiting T cell activations when PMA/Iono was the stimulus, as PMA/Iono is a known PKC agonist and triggers T cell activation downstream of the TCR signaling cascade. This contrasts with the other mitogens tested that stimulate through the TCR, and where activation was severely impeded by Dasatinib. Interestingly, when Dasatinib was used as an inhibitor in our latency reversal reactions, a marked reduction in induced virus was detected in culture supernatants, approaching ~40% (Figure 3-6G). This indicates the importance of signaling through the TCR which is likely the mechanisms employed by MDDC pulsed with ACT-VEC.

While contact and signaling through the TCR appear important, inhibition of JAK-STAT and TNFR mediated signal transduction did not significantly reduce ACT-VEC mediated latency reversal in our hands, suggesting that cell secreted factors alone are not responsible for the observed latency reversal (Data not shown). Additional support for this is shown by ACT-VECs comparatively lower capacity to induce IFN-γ, TNF-a and IL-2 production from activated CD4 T cells compared to the polyclonal stimulator PMA/Iono (Data not shown). The latter being a poor latency reversal agent in these studies.
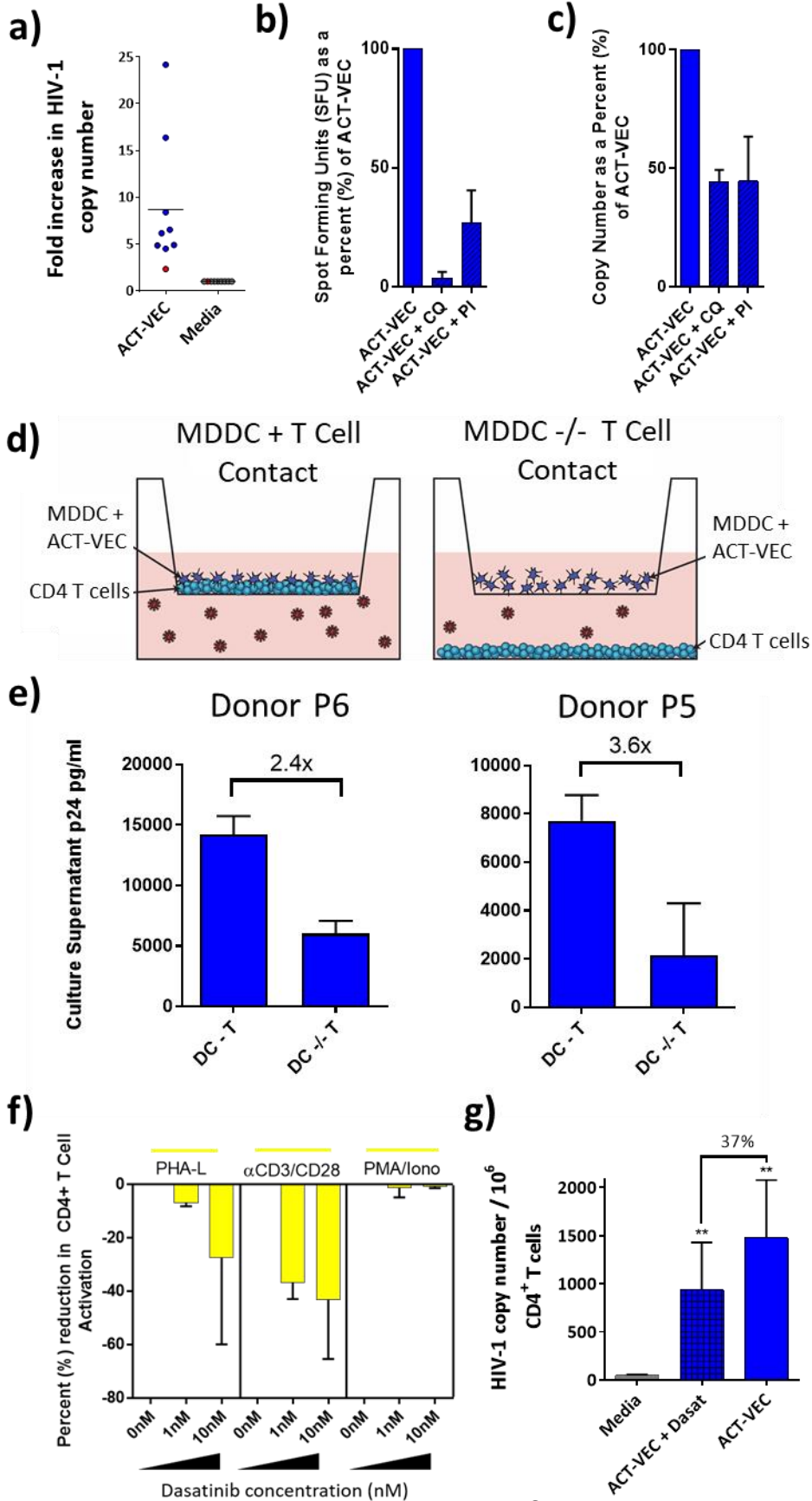
Figure 3-6: MDDC presentation of ACT-VEC to T cells induces replication competent virus though contact dependent mechanisms.

(a) PBMC from volunteers treated with cART containing a protease inhibitor induced the lowest fold change in viral reactivation by qRT-PCR. **(b)** MDDCs were pulsed with ACT-VEC in the presence or absence of either the protease inhibitor, Pepstatin A, or the lysosomotropic agent, Chloroquine (CQ), and subsequently co-cultured with autologous CD4 T cells. IFN-γ ELISpot SFUs were used to evaluate the percent (%) change in activation between conditions. **(c)** After 3 days in culture, supernatants were collected and viral copy number determined via qRT-PCR. **(d)** To evaluate if MDDC-CD4 T cell contact was necessary for HIV latency reversal, the MDDC-CD4 T cells were either cocultured in the apical insert of the Spin-X columns or separated by seeding them in the basolateral and apical chambers of the columns, respectively. **(e)** After MDDC were pulsed with ACT-VEC and the MDDC used to stimulate autologous CD4 T cells, induced virus in culture supernatants was quantified by p24 ELISA. **(f)** To evaluate the effect on latency reversal by stimulating through the TCR, we used an inhibitor of the TCR signaling pathway, Dasatinib. To determine the best concentration of Dasatinib to use in latency reversal studies, we titrated Dasatinib on CD4 T cells at varying concentrations (0nM, 1nM, 10nM) prior to activation with either PHA-L, αCD3/CD28, or PMA/Iono. Percent activation was determined via flow cytometry. **(g)** ACT-VEC-pulsed MDDCs were co-cultured with CD4 T cells in the presence or absence of Dasatinib, and supernatants were analyzed for viral RNA concentrations (copy number +/- SEM) after 24 hours.

3.4 Discussion

Development of an HIV-1 cure is a significant challenge. Currently, only the Berlin patient has been cured of HIV-1, although it has recently been reported that a London patient and Düsseldorf patient have also been described to be in HIV-1 remission[46–48]. However, the myeloablation/stem cell transplantation therapy used to achieve these remarkable feats are impractical for global roll out or use in the cART era[46]. Regardless, the addition of the London and Düsseldorf patient does add further credibility to the HIV cure effort and also adds supportive evidence that the cure seen in the Berlin patient was not an anomaly, despite the lack of HIV eradication seen in other clinical examples such as the Mississippi Child and the Boston patients. Alternative approaches to HIV cure have been based on *in vitro* stimulation of latently HIV infected cell lines and primary T cells using a diverse array of chemotherapeutic and immunomodulatory agents[49,50]. However, the initial promising *in vitro* effects of HDACi and PKC agonists on latency reversal have parlayed to less effective activation in primary CD4 T cells of cART-treated volunteers; resulting in subtle to no changes in the size of the latent HIV pool.

For full HIV latency reversal, an LRA must overcome key epigenetic barriers. The latent HIV genome is typically integrated into heterochromatin or genomic regions of limited transcriptional activity. TCR mediated stimulation induces a complex signaling cascade leading to increased levels of transcriptional factors NF-kB, NFAT, AP-1, p-TEFb, and Ets-1 as well as other host factors, all of which interact with the promoter/enhancer elements in the LTR to activate, enhance and/or elongate HIV-1 mRNA transcription[51,52]. In the studies presented herein, we stimulated the T cells through HIV-specific TCRs using a targeted approach employing MDDC and a susceptible HIV VLP formulation. We chose AC-cART samples for this study as the minimal genetic diversity and lower reservoir size is likely to play a significant role within immunotherapeutic cure strategies. This differs significantly from most cure studies that evaluated latency reversal in samples derived from individuals diagnosed and treated at chronic stage of infection, where reservoirs and diversity are going to be larger. Our HIV-specific, CD4 T cell-targeted approach was based on several studies suggesting that these HIV-specific T cells may be the primary "fuel" for HIV production, especially during acute/early infection[15,16]. HIV antigens, however would only be an effective transcriptional shock if CD4 T cells expressing HIV-specific TCRs served as a significant

reservoir for latent HIV, which is indeed the phenomenon that we observed. Simply put, ACT-VEC+MDDC resulted in the production and release of HIV from CD4 T cells of volunteers receiving cART during early infection (AC-cART). This latency reversal and release of HIV-1 by our immunotherapy was significantly greater than that observed when the same AC-cART CD4 T cells were treated with common recall antigens, HDACi and even potent mitogens.

Using deep gene sequencing of the induced, propagating virus, we were able to estimate the numbers of latently infected cells that were inducible within patient samples. Our results suggest there are ~14 CD4 T cells per million with infectious but latent HIV provirus that can be stimulated to produce HIV in our acute samples. This falls within current estimates in the literature of between 1-60 CD4 T cells /million harboring latent but potentially infectious HIV provirus during stable cART[38–40]. Interestingly, a study by Baxter *et al* demonstrated that in a FISH/Flow assay, the median frequency of PMA/Iono inducible cells harboring latent HIV was 3.56 CD4 T cells/10^6, which is substantially less than our calculated 14 cells/million and agrees with the low level reactivation that we see in these studies[53]. In the future, studies using more powerful technologies, such as the before mentioned FISH/Flow, and newer assays that can discriminate between intact and defective forms of virus, such as the Intact Proviral DNA Assay (IPDA), would likely shed important insights into the effectiveness of our shock approach while more accurately characterizing the reservoir size[54].

While the fate of the cellular reservoir upon viral reactivation is unknown, activation of HIV-specific memory CD4 T cells by ACT-VEC could provide a strong T helper response for CTL or antibody-based immune control of this reactivated latent HIV. Therefore, it is important to note that we have previously shown ACT-VEC induced cell-mediated cytotoxic Granzyme B responses in these AC-cART treated volunteer PBMC[27]. In the context of immunotherapeutic cure, particularly in patients treated during acute/early infection, an antiviral cytotoxic response induced by ACT-VEC may be very effective in eliminating the relatively homogenous HIV-1 presented by infected cells via MHC I. Thus, ACT-VEC, delivered via adoptive transfer of *in vitro* generated, antigen-loaded DCs, or *in situ* targeting of DCs could prime for a possible "kill" along with the observed "kick" that we see. Over the years, a number of studies have used DC-based approaches to present HIV-1 antigens in the non-human primates and in clinical trials to enhance

an immune activated "kill" of the cell reservoir and/or residual virus[55–59]. Notable reductions in viral RNA and DNA levels were observed in the cells from various anatomical compartments of the vaccinated animals, a finding that has yet to be substantiated in human trials. However, these studies were focused on a "kill" by CD8+ T cytotoxic lymphocytes rather than exploring how DC mediated presentation of these SIV antigens may have activated virus-specific CD4 T cells and thereby triggering latency reversal.

While these studies were conducted using samples from patients, cART-treated at acute stage of subtype B infection, it should be noted that the vast majority of individuals are diagnosed much later in infection. During chronic stage of infection, untreated individuals would have been exposed to a plethora of opportunistic infections which might change the TCR demographics of cells harboring latent provirus. Equally, it should be considered that subtype B infection accounts for a considerably smaller proportion of HIV infection, with subtype C constituting the majority of the global epidemic. Therefore, in the future it would be interesting to evaluate the effectiveness of ACT-VEC at mediating a latency reversing shock in both chronic and acute patient samples as well as evaluating the effects of ACT-VEC at triggering latency reversal of differing subtypes of HIV.

In conclusion, we demonstrate that ACT-VEC efficiently reactivates transcriptionally silent HIV-1 from CD4 T cells of patients treated at acute/early infection. Furthermore, we can show viral translation occurs and that the released virus is replication competent. Our study also demonstrates that this immunotherapy is targeted to HIV-specific CD4 T cells, which may house a large proportion of the latent HIV reservoir. Taken together, this approach establishes ACT-VEC as a highly promising immunotherapeutic LRA. Future planned *in vitro*, *in vivo* non-human primate and human clinical studies will suitably address whether ACT-VEC can indeed cause reservoir reduction/elimination and whether ACT-VEC can induce protective humoral and cytotoxic T cell responses *in vivo*.
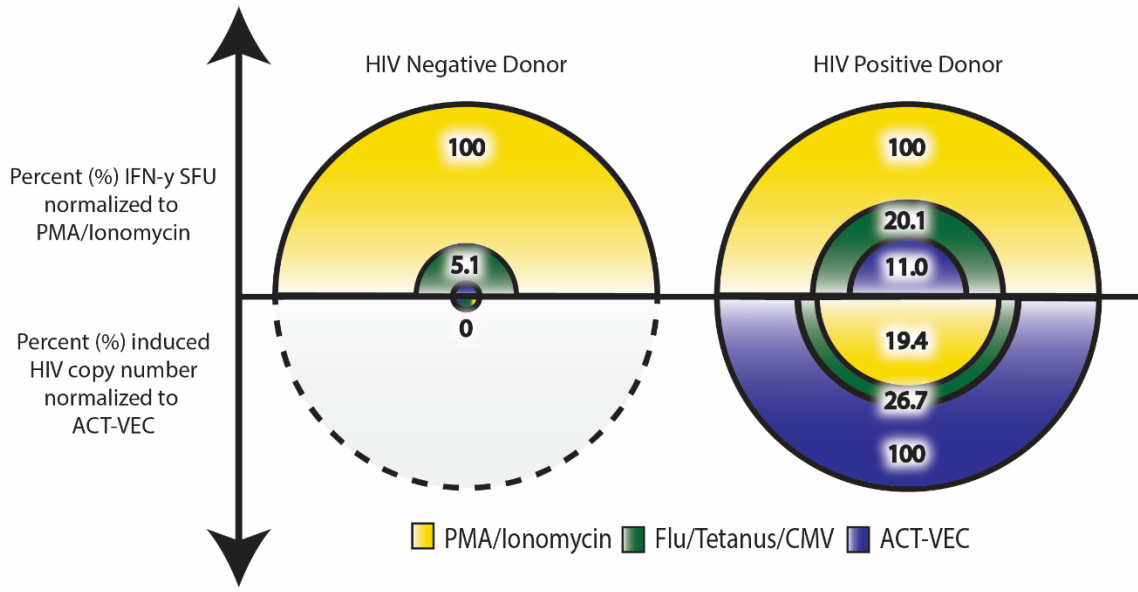
Figure 3-7: Schematic representation of latency reversal in human acute/early CD4 T cells.

Representation of the frequencies (%) of CD4 T cell activation and the quantities of induced HIV-1 virus detected in culture supernatants using PMA/Iono (▢), Flu M1/Tetanus Toxoid/CMV peptide pool (▢) and ACT-VEC (▢). Frequencies of induced virus (below the x-axis) and activated CD4 T cells (above the x-axis) are normalized to the condition eliciting the greatest response (i.e. ACT-VEC for induced virus and PMA/Iono for activated CD4 T cells). The schematic demonstrates that ACT-VEC is a stronger latency reversal agent than non-specific stimuli whilst being substantially less antigenic.

3.5 Materials and Methods

3.5.1 Experimental Design

The activator vector (ACT-VEC) LR agent was developed as an HIV-1 curative strategy. Based on previous studies suggesting that HIV-1 preferentially establishes a latent reservoir within antigen-specific CD4 T cells, we hypothesized that an HIV-1 antigenic formulation containing the epitopes that could be presented to cognate TCR bearing T cells, would be a promising transcriptional shock tactic. We therefore generated a highly diverse, polyvalent virus like particle (VLP) formulation by combining the HIV quasi-species from 5 chronic HIV-infected volunteer's plasma samples, taken immediately prior to cART initiation. The objectives of this study were succinct. In the first instance, we aimed to demonstrate a proof of concept that ACT-VEC could efficiently trigger viral LR in purified human CD4 T cells from patients receiving cART and show that the reservoir was indeed concentrated in HIV-specific memory CD4 T cells. Furthermore, we aimed to demonstrate that ACT-VEC induces replication competent virus i.e. the source of recrudescing virus upon cART cessation. Finally, we sought to compare the efficacy of ACT-VEC mediated LR to that induced by other LRAs such as HDACi and PKC agonists. To achieve the objectives of the study, we recruited cART-treated individuals that were diagnosed with HIV at acute/early stage of infection and who were able to durably suppress viremia. The recruited individuals consented to supply a large blood draw from which we could evaluate ACT-VEC LR on CD4 T cells *ex vivo*.

3.5.2 Ethics statement

HIV-infected individuals were recruited from the HIV adult clinical services at St Mary's Hospital (Imperial College NHS trust), through a protocol approved by the NHS Health Research Authority (protocol number: 14SM1988). Peripheral blood was obtained from infected patient volunteers who had suppression of viremia to <50 copies HIV-1 RNA/mL for > 6 months on ART. All study subjects used in this manuscript were adults and gave prior written informed consent.

3.5.3 Virus like particle (VLP) latency reversal agents and their production

A highly diverse virus-like particle (VLP) formulation was constructed from the serum of HIV+ subtype B patient volunteers, identified and receiving cART at chronic phase of infection (isolated

from patients infected for more than 1 year). Briefly, viral RNA was isolated from a banked plasma sample, prior to cART initiation, using a viral RNA isolation kit (Qiagen, USA). Viral RNA was reverse transcribed (Agilent Technologies, USA) to full length HIV-1 proviral cDNA as two fragments using two reverse primers to generate the 4561 bp 5' ($\Delta\psi$ region - Integrase) and 4880 bp 3' fragment (integrase -3'LTR) regions. The cDNA fragments were then utilized in a nested PCR protocol using 5' and 3' primer sets to create 5' and 3' overlapping fragments. Both the 5' and 3' overlapping primers for the two genomic halves contained mutations generating a AAH > RRK substitution in the Integrase (IN) coding region and destroys IN activity[27]. The 5' fragment ($\Delta\psi$ region - Integrase) was amplified with 5' primer that disrupts the ψ packaging region, preventing genomic RNA encapsidation[27]. The two subsequent (5', 4378 bp and 3', 4628 bp) half genome fragments were transfected into *Saccharomyces cerevisiae* in a 1:1 ratio (wt/wt) with 2μg SacII linearized plasmid, pRECΔgag-U3/URA3. Resulting yeast colonies resulting from a triple recombination/gap repair into the pRECΔgag-U3/URA3 to produce pREC_nfl_UH8.dS1.IN$_{AAH>RRK}$ were selected on complete medium lacking leucine (C–Leu) plates supplemented with fluoroorotic acid (FOA). It is important to note that the pREC_nfl_UH8.dS1.IN$_{AAH>RRK}$ also lacks the 5' LTR and produces defective gRNA that cannot be packaged (due lack of ψ) and cannot serve as a template for reverse transcription (due to lack of 5' LTR). The recombined plasmid vectors were isolated by yeast mini-prep, transformed into bacteria, purified using Maxiprep (Qiagen, CA) and then used to transfect 293T cells with Fugene 6 transfection reagent (3:1 vol/wt) (Promega, USA) to produce VLPs. Viral VLPs were first purified in cell-free supernatant by centrifugation through 100 KDa MWCO centrifuge tubes (Amicon, USA), before ultracentrifugation at 100,000g (Beckman, USA) with the resulting VLP pellet re-suspended in sterile PBS. To verify VLP particle production, the formulation was confirmed by dynamic light scattering, transmission electron microscopy, p24 production, radioactive reverse transcriptase assay for presence of Rev and Tat, Western blot and cell-cell fusion co-receptor tropism assay VERITROP for presence of Env and its functionality as described previously[27]. Following these safety and purity tests, VLPs are referred to as ACT-VEC.

3.5.4 Isolation of resting CD4 T lymphocytes and Monocyte Derived Dendritic Cells (MDDC)

Peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation. CD4 T lymphocytes were enriched by negative depletion (CD4 T cell Isolation Kit, Miltenyi Biotec) using magnetic microbeads. The purity of these cells was analyzed by flow cytometry using a LSR II (BD Biosciences, USA) and FlowJo software (Treestar). To obtain immature DCs, PBMC were cultured for 2 hours in a T-75 tissue culture flask (Sarstedt, Ca) and allowed to plastic adhere for 2 hours in a 5% CO_2 incubator at 37˚C. The adherent monocytes were washed gently with complete RPMI to remove non-adherent cells. Isolated monocytes were then differentiated into monocyte derived dendritic cells (MDDC) by culturing in complete RPMI (10%FCS + 2mM L-Glutamine) supplemented with GM-CSF and IL-4 (1,000 and 500 U/mL respectively) in T-75 flasks for 6 days with 50% of the media replenished with fresh complete RPMI (IL-4 + GM-CSF) every 3 days.

3.5.5 Latency reversal assays

Purified CD4 T cells were co-cultured overnight with day 7, antigen-pulsed, autologous MDDC at a ratio of 1 DC per 4 T-cells. Antigen pulsing consisted of overnight stimulation of MDDC with either 1) media, 2) 5 µg/mL (based on p24) heterologous ACT-VEC, 3) recall antigen cocktail CTF [250 ng/peptide/mL Flu M2 protein (bei Resources, USA), 2 µg/mL Tetanus toxoid (Statens Serum Institut, DK) and 5 µg/mL pp65 HCMV peptide pool (NIH AIDS Reagent Program, USA)]. On the day of MDDC-T cell co-culture either, 1/5000 phorbol 12-myristate 13-acetate (PMA) + Ionomycin (Iono) (ebioscience, Ca), 10 nM PKC agonist Bryostatin-1 (Sigma, USA) or 335 nM Histone Deacetylase inhibitor (HDACi) Vorinostat (Caymen Chemicals, USA), 40 nM Romidepsin (Cedar lane labs, CA) or 30 nM Panobinosat (Selleckchem), was added to the remaining un-pulsed MDDC-T cell co-cultures to serve as controls and competitor LRAs. All HDACi and PKC agonists were used at previously published concentrations known to upregulate viral RNA transcripts in primary T cells and cell lines. The fusion inhibitor, Enfuvirtide (T-20) was added after 24 hours to all co-cultures at a concentration of 10 µM to block any re-infection of T cells by the reactivated

latent virus. In all cases, stimulated co-cultures were loaded onto IFN-γ ELISpot plates to assess T cell activation.

For studies involving measurements of LR occurring as a result of cell-cell contact or soluble secreted factors, ACT-VEC pulsed MDDC were pulsed overnight before, washing and incubating with/without autologous CD4 T cells in the apical or basolateral chamber of a 0.4μm Spin-X Columns (Sigma, Ca). For viral propagation studies, MOLT4 CCR5 cells were added to the bottom chamber of the Spin-X column to allow the propagation of replication competent virus released from patient CD4 T cells. Supernatants were harvested every 3 days and HIV-1 infection was determined by p24 ELISA (NCI Frederick, USA) and assessed by qRT-PCR. To evaluate the impact of various signal transduction pathways on ACT-VEC mediated latency reversal, 10nM Dasatinib (Sigma, Ca), 40nM Methotrexate (Sigma, Ca) and 1.7μM R7050 (Santa Cruz, Ca) were added to CD4T cells 6h prior to coculture with ACT-VEC pulsed MDDC. For studies involving the titration of Dasatinib and stimulation with the T cell mitogens, PMA/Iono, PHA-L, and αCD3/αCD28, purified CD4 T cells were culture in the presence of inhibitor overnight prior to flow cytometric analysis. For inhibition studies involving Pepstatin A (2μM, Sigma, USA) and Chloroquine (100μM, Sigma, USA), inhibitors were added to MDDC overnight prior to overnight pulsing with ACT-VEC.

3.5.6 IFN-γ antigen-specific ELISpot assays

Human IFN-γ enzyme-linked immunosorbent spot (ELISpot) assays (Mabtech, USA) were carried out on MDDC-CD4 T cell co-cultures as per the manufacturer's instructions. Briefly, anti-IFN-γ pre-coated plates were washed with sterile PBS and then blocked for 30 min using complete RPMI. Plates were again washed before addition of 1×10^6 cells/mL CD4 T cell. The MDDC stimulated CD4 T cells were then incubated for 16 h to assess the number of HIV-specific CD4 T cells. Unstimulated and PMA/Iono (eBioscience, Ca)-stimulated cells, diluted 1/500 (v/v) in media, served as controls. To detect spots, biotinylated anti-IFN-γ antibody was added at 1 μg/mL for 2 h before washing and incubating with streptavidin-HRP for 1 h. Plates were washed as described above, and 100 μl/well of TMB substrate was added.

3.5.7 Flow Cytometry

For latency reversal in J-Lat 6.3 (AIDS Reagent Program) cell studies, cells were seeded overnight, in a 6 well tissue culture plate, at 2.5 million cells/well along with the various stimuli. Samples were washed three times using FACs buffer (2.5% FCS in PBS) and then fixed in 1.5% methanol-free paraformaldehyde (Polysciences, USA) in PBS. Samples were analyzed for GFP expression on a FACS LSR II instrument with FACS Diva software. Data analysis was performed with FlowJo (Treestar Inc., OR, USA).

For evaluation of MDDC primary cell maturation, monocytes from HIV negative donors were purified via plastic adherence and matured into MDDCs using IL-4 and GM-CSF as described previously. MDDCs were pulsed using either ACT-VEC VLP, CTF, HIV Consensus B Env/Gag/Pol peptide pools (2 µg/peptide/mL; NIH AIDS Reagent Program, USA), or remained un-pulsed for the negative control. Cells were stained for the cellular maturation markers CD11c (Clone: 3.9, eFluoro450; eBioScience, Ca), CD209 (Clone: eB-h209, PE-Cy7; eBioScience, Ca), CD40 (Clone: 5C3, BV711; BioLegend, USA), CD80 (Clone: L307.4, BD Pharmingen, USA), and CD83 (Clone: HB15e, FITC; BioLegend, USA). Samples were fixed and analyzed as described previously.

For polyfunctional CD4 T cell stimulation studies, purified CD4 T cells were pulsed with the various stimuli for 6h and stained using anti-human CD3 (Clone: SP34-2, APC-H7; BioLegend, USA) and anti-human CD4 (Clone: L200, BV711; BioLegend, USA), anti-human IFN-γ (Clone: 4S.B3, FITC; BioLegend, USA), anti-human IL-2 (Clone: MQ1-17H12, PE-Dazzle 594; BioLegend, USA), and anti-human TNF-α (Clone: MAb11, APC; BD Bioscience, USA). Samples were fixed and analyzed as described previously.

3.5.8 Detection and quantification of cell-free supernatant HIV-1 mRNA.

After 18h of stimulation, HIV-1 mRNA was extracted using a Viral RNA extraction kit (Qiagen, CA) from 0.2 mL of cell-free culture supernatant, which corresponds to 200,000 purified LRA-activated CD4 T cells. cDNA synthesis was performed using AccuScript High fidelity RT-PCR system kit (AccuScript, USA). Real-time PCR was performed using SensiFAST SYBR™ No-ROX kit (Bioline, USA) on a Rotorgene 6000 Real-Time PCR machine (Corbett Research, USA) or QuantStudio5

(Applied Biosystems, Ca). Primers sets are listed in table S1. A 2-step cycling condition was carried out as follows: 1) 95°C for 2min, 2) 30 cycles of 95°C for 5s and then 63°C for 30s. Molecular standard curves were generated using 1/10 serial dilutions of in house prepared pREC_5'LTR-SBF-1 plasmid which has a known concentration and contains an intact 5'LTR and gag sequence from clade B NL4-3. The human TBP (TATA-box binding protein) primer set and control plasmid was used as an endogenous control (a kind gift from Dr Jimmy Dikeakos).

3.5.9 Verification of LRA-induced HIV-1 RNA by deep gene sequencing.

3.5.9.1 Library Preparation for Next Generation Illumina Sequencing:

Amplicon products from the 5'LTR to Gag were generated from cDNA by external-nested PCR using a hot start polymerase (Platinum Taq, Invitrogen). External PCR primers included 5LTRPA and GAGREV. For the nested, PCR primers with Illumina overhang adapter sequences were used: MiS_PBSdt-F and MiS_GAGdt-R. Amplicons were further produced for the V2-V3 region of Env using the external PCR primers EnvB and ED14. The nested PCR utilized primers with Illumina adapter sequences E80_IlluminaTag and E125_IlluminaTag. PCR cycle conditions were 95°C for 3min, followed by 35 cycles of 95°C for 30s, 55°C for 30s and 72°C for 1min (external) or 45s nested, and a final extension of 72°C for 10min. The PCR products were run on a 1% agarose gel at 100V for 45min to verify products of 350 bp (5' LTR-Gag) or 481 bp (Env). Products of the appropriate band size were excised from agarose gel using a QIAquick Gel Extraction Kit. Concentrations were determined using a NanoDrop 2000c Spectrophotometer (Thermo Fischer, USA).

3.5.9.2 Index PCR for the Illumina MySeq system:

The Nextera XT Index kit system (NexteraXT Index Kit v2 Set A, Illumina) was used to attach the Illumina dual-index sequence adapters to the amplified and purified PCR products using a limited cycle index PCR (KAPA HiFi HotStart, Kappa Enzymes). PCR cycle conditions were 95°C for 2min, followed by 35 cycles of 95°C for 30s, 55°C for 30s and 72°C for 45s, and a final extension of 72°C for 10min. The PCR products were run on a 1% agarose gel at 100V for 45min.

3.5.9.3 Sample library purification and quantification:

All PCR products were purified using Magnetic beads (AMPure XP, Berkin Elmer) with a bead:DNA ratio of 1:1. The DNA was eluted in 50 ul 10mM Tris (pH 8.5). Libraries were then flourometrically quantified (QuantIT PicoGreen, Invitrogen), normalized to 500ng and pooled together.

3.5.9.4 Illumina Sequencing:

The pooled samples were processed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca) using the Illumina MiSeq (Illumina, Inc., San Diego, CA). The library was run on an Agilent (Agilent Technologies Inc., Palo Alto, CA) High Sensitivity DNA Bioanalyzer chip (Caliper Life Sciences, Mountain View, CA) to assess size distribution and qRT-PCR (Kapa Biosystems, Inc., Wilmington, MA) was used to assess the quantity. The library was diluted to 4 nM and sequenced (according to the Illumina protocol Preparing Libraries for Sequencing on the MiSeq, Rev. C) on an Illumina MiSeq using a 2 x 300 paired end run with 10% PhiX.

3.5.9.5 Data analysis:

Raw FASTQ paired reads (R1 and R2) files were quality checked with FastQC to visually evaluate the presence of Nextera paired-end adapters and to use a successive trimming strategy for detection and elimination of low quality base calls. Adapter removal and trimming were performed with Trimmomatic using the SLIDINGWINDOW option (cutting the remaining sequence once the average quality within a window of 4 nucleotides falls below a threshold of 20). The trimmed FASTQ outputs were then utilized for mapping against HIV reference genome HXB2 (GenBank accession number K03455) using bowtie2 with local alignment and using an iterative re-mapping strategy to adapt the reference genome to the sample. Finally, the mapping results were visualized with IGV for detection of 5'LTR and the dS.1 mutation.

For *env* sequence analysis of proviral and induced virus, we analyzed a 277-nucleotide long fragment (HXB2, coordinates 6845-7121), which we subsequently collapsed to identify the unique sequences using the MiCall pipeline (https://github.com/cfe-lab/MiCall). The multiplicity values of the sequences were used to calculate the 1% cut off for the total number of input sequences for each sample. The resulting sequences were then defined as "unique sequences".

The unique sequences were analyzed using the Los Alamos National Laboratory (LANL) Neighbor TreeMaker tool. The LANL database was used to acquire reference sequences. The output style was modified for clarity, to show symbols using Powerpoint.

3.5.10 Statistical analysis:

Mann-Whitney non-parametric, or Wilcoxon matched pairs signed rank test was used to determine intra- and intersample statistical significance where indicated. We considered $P > 0.05$ to be statistically significant. Correlations between sample conditions were evaluated using a two-tailed Spearman Rank Correlation where indicated. HIV-1-infected adult volunteers meeting the criteria for sustained viral load suppression (>6 months prior to sample donation) and free from any viral blips were enrolled. As samples from all patients were handled in the same way, there was no randomization or blinding.

3.6 Acknowledgments

3.7 Competing Interests

The authors declare no competing interests.

Figure 3-8S: Schematic of latency reversal studies and ACT-VEC preparation.

The HIV+ volunteers were recruited based on early HIV diagnosis and cART treatment. Eligible volunteers had to have durable suppression of viral loads to below 50 HIV copies/ml for the six months preceding enrollment. Peripheral blood mononuclear cells (PBMC) were isolated and immediately cryopreserved. On study day 0, PBMC were defrosted and monocyte derived dendritic cells (MDDC) generated by 6 days of culture in IL-4 and GM-CSF. On day 6, the MDDC were pulsed with stimuli and autologous PBMC were defrosted and rested overnight. On day 7, the PBMC were used to isolate CD4 T cells and co-cultured with the pulsed MDDC. **(a)** The MDDC-CD4 T cells were then further cultured to determine viral RNA concentrations in culture supernatants and for T cell activation by ELISpot and flow cytometry. The activator vector (ACT-VEC) virus like particles (VLPs) were generated by first isolating HIV RNA from the plasma of HIV+

volunteers. The HIV genome was PCR amplified in two overlapping halves and recombined in yeast by gap/repair using an in-house developed recombination vector, pREC HIV-Δ/URA3. The PCR process deletes the 5'LTR, inserts extensive nucleotide substitutions into stem loop 1 (SL1) of the RNA packaging sequence (ψ) and disrupts integrase activity. Positive recombination results in yeast colony formation under 5-Fluoroorotic acid selection on C-leu-URA agar plates. The DNA is extracted and amplified in bacteria before being used to transfect HEK293T cell. **(b)** After 72h transient transfection, ACT-VEC VLPs are harvested and purified for latency reversal studies.
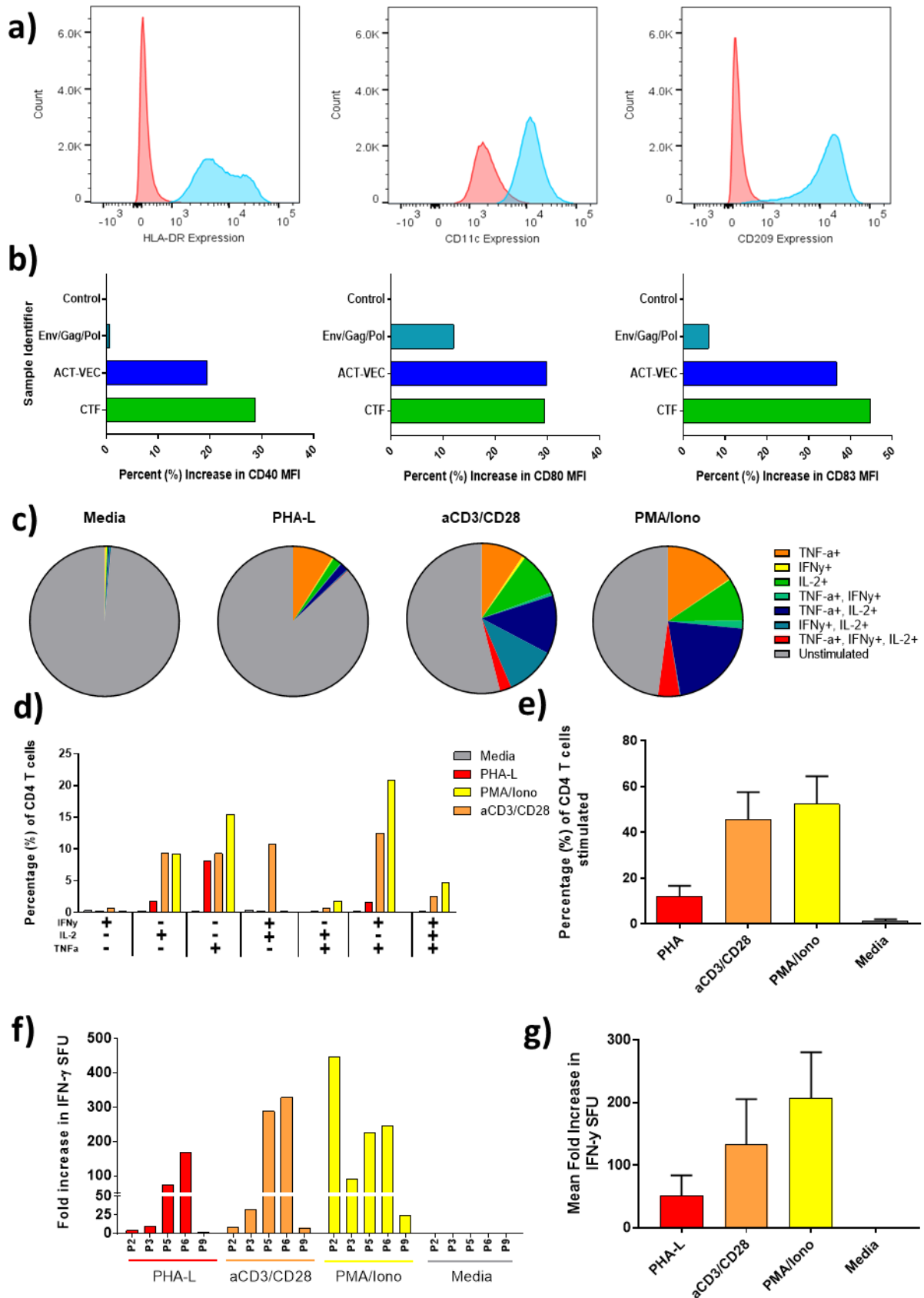
Figure 3-9S: Determination of MDDC maturation and polyfunctional CD4 T cell induction in healthy donors.

(**a**) Plastic adhered monocytes, differentiated in the presence of IL-4 and GM-CSF, were evaluated for differentiation into MDDCs using HLA-DR, CD11c and CD209 expression via flow cytometry (n=3). (**b**) MDDCs were pulsed with CTF, ACT-VEC, or Env/Gag/Pol peptides and tested for maturation using CD40, CD80, and CD83. (**c**) CD4 T cells, purified by magnetic bead isolation of PBMCs, were stimulated with either PMA/Iono, PHA-L, or αCD3/CD28 to determine optimal stimulation conditions for use in latency reversal assays (n=3). Purified CD4 cells were run on a Flow Cytometer and gated using CD3 and CD4 and their activation determined by positive staining for IL-2, IFN-γ, and TNF-α. (**d**) Stimulated CD4 T cell polyfunctionality was assessed by analyzing mono, dual, or triple cytokine function. (**e**) The mean percentages of total CD4 T cells expressing one, two or three functions is shown. (**f**) Healthy donor cells (n=3) were analyzed via IFN-γ ELISpot to reaffirm the activation potency of PHA-L, αCD3/CD28, and PMA/Iono conditions. (**g**) Grouped samples were used to determine stimulation mean (±SEM) effects on antigenicity.
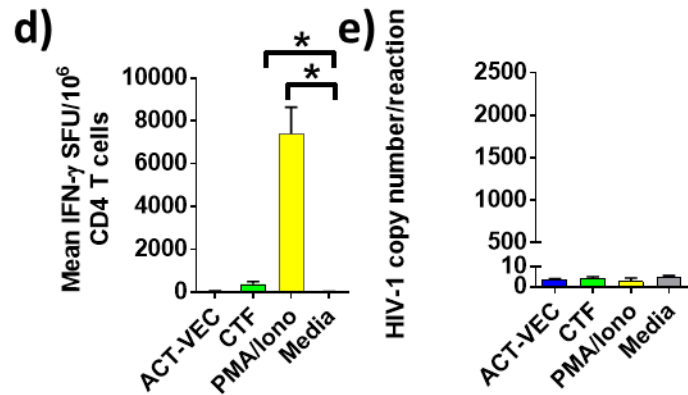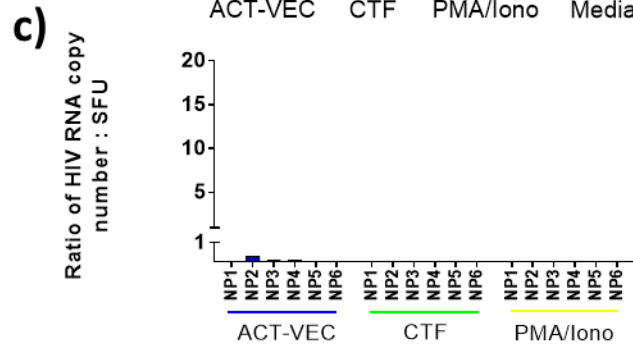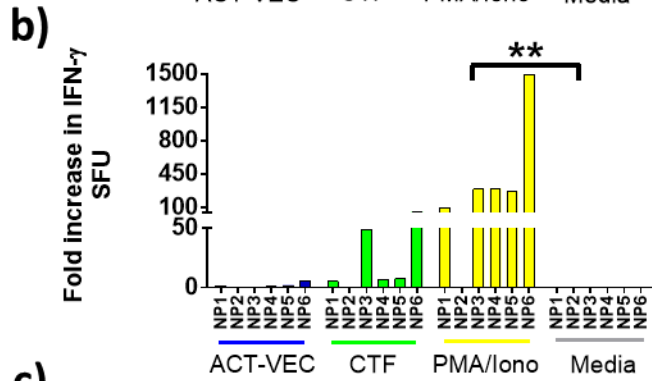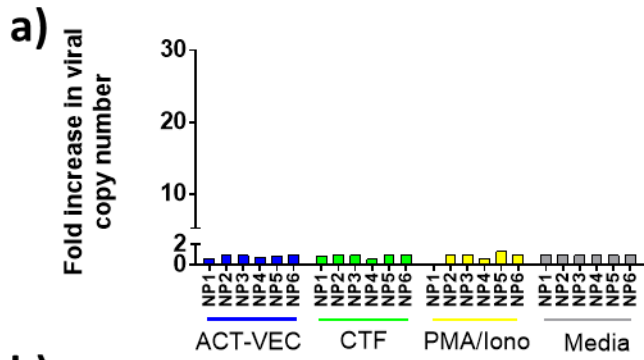
Figure 3-10S: Healthy donors lack contaminating viral RNA and exhibit varying degrees of CD4 T cell activation in response to stimuli.

MDDC+CD4 T cell cultures from healthy individuals (n=6) were stimulated with ACT-VEC (5ug / mL), a cocktail of Flu M1, Tetanus toxoid, CMV peptide pool (CTF), or PMA/Ionomycin. (**a and e**) Culture supernatants were collected after three days of culturing and tested for the presence of viral RNA via qRT-PCR. Due to the lack of HIV-infection within these donors, viral RNA was undetectable (<5.6 copies/mL) in all stimulation conditions. (**b and d**) CD4 T cells that were co-cultured with autologous MDDCs were tested for activation via IFN-γ ELISpot. ACT-VEC exhibited low/no activation in most conditions, as compared to the global activator PMA/Iono (**p=0.005), or the recall antigen cocktail CTF. (**c**) The ratio between HIV copy number and activation was determined. Grouped samples were used to determine the mean effect (±SEM) of the stimulations on antigenicity and induced copy number with statistical significance ascertained by Mann-Whitney unpaired t-test.
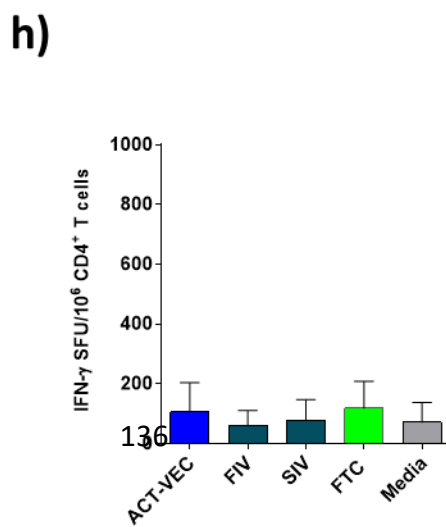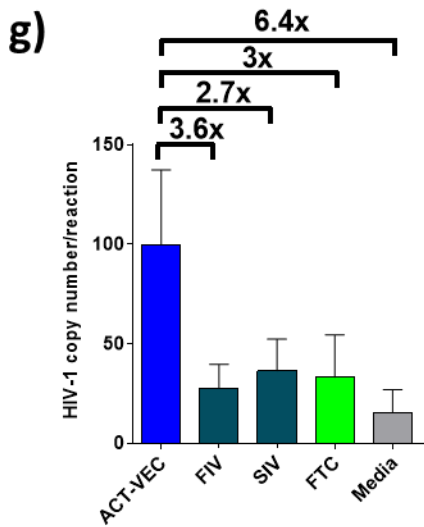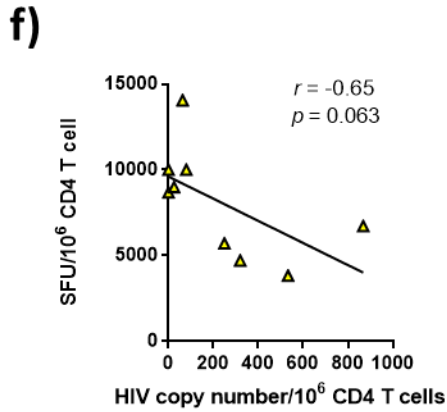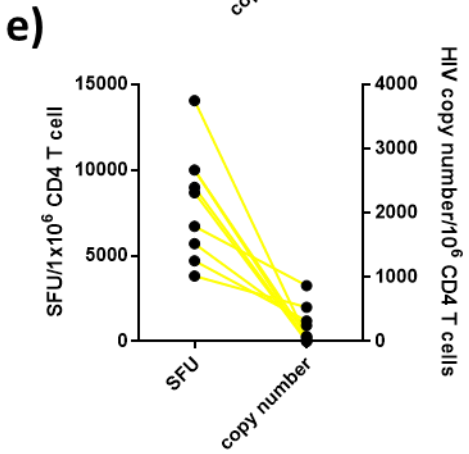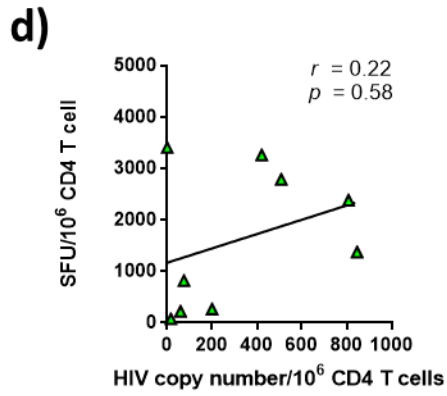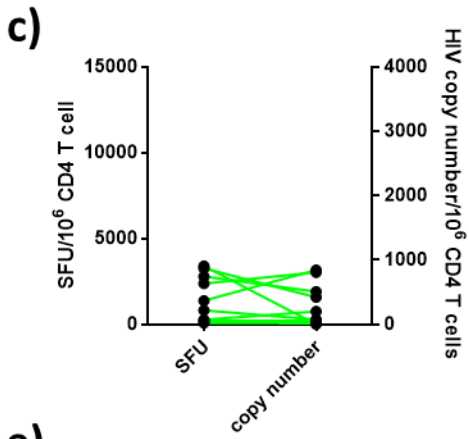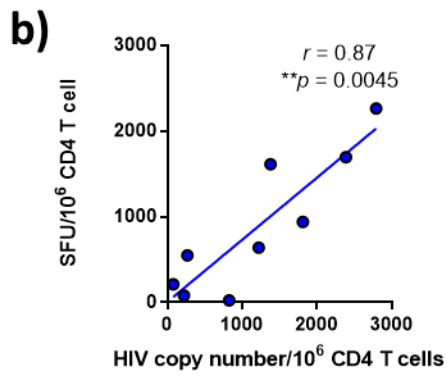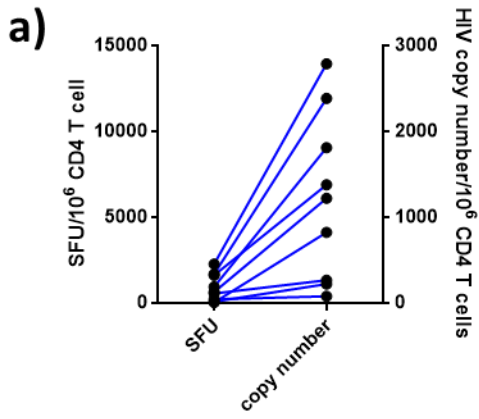
Figure 3-11S: An extended comparison of various stimulations to induce HIV and activate CD4 T cells.

(**a,c,e**) The ability of the various latency reversal agents (ACT-VEC, FTC and PMA/Iono) to stimulate HIV induction and detection in culture supernatants was compared against its ability to stimulate CD4 T cells. (**b,d,f**) Correlations between induced virus concentrations and CD4 T activations were then assessed. Induced HIV was quantified within 3-day culture supernatants of stimuli-pulsed monocyte derived dendritic cells (MDDC) co-cultured with purified CD4 T cells and using qRT-PCR. To quantify induced virus, a primer set specific for the 5'LTR was employed. To determine the immune-stimulatory potential of the stimuli, IFN-γ ELISpot was used, and the co-cultured MDDC-CD4 T cells incubated for 19h to determine recall or non-specific responses to the stimuli. Results represent data from all 9 volunteers. We further compared ACT-VEC VLPs to inert SIVmac251 and FIV viral particles. The FIV in the pREC plasmid backbone was kindly provided by Dr Ryan Troyer while SIVmac251 viral particles were generated in house according to the same methodology as ACT-VEC. The SIVmac251particles were recombined into a specially synthesized pRECΔgag-U3 vector with flanking regions of homology in the 5' packaging sequence (ψ) and 3'LTR end. As with ACT-VEC, the resulting SIVmac251 lacked a 5'LTR but in difference to ACT-VEC does contain a functional genomic packaging sequence. The ACT-VEC, FIV and SIV particles were used to pulse MDDC prior to 72h coculture with purified CD4 T cells. (**a**) Induced virus was quantified in culture supernatants by qRT-PCR. Due to the scarcity of the HIV infected donor samples, this experiment was only performed once with two donors. Assay cutoffs for ELISpot and qRT-PCR are 50 SFU/10^6 CD4 T cells and 30 viral copies/reaction. Results shown are group means +/- SEM. (**g,h**) HIV-1 copy number and antigenicity was ascertained for related, non-HIV VLPs using the standard MDDC-T cell co-culture assay, and were subsequently compared to ACT-VEC.
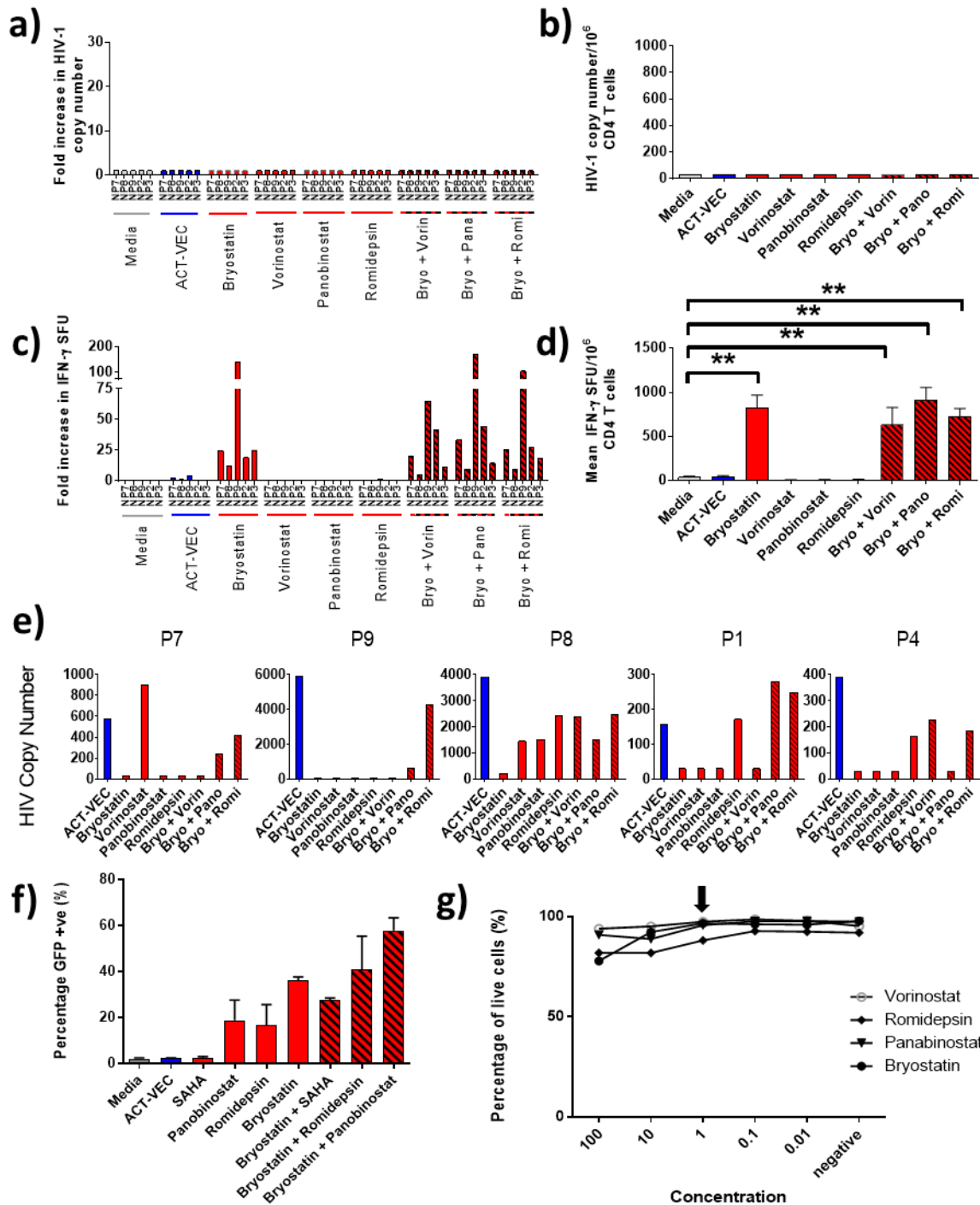
Figure 3-12S: ACT-VEC specifically activates HIV-infected donor samples when compared to alternative LRAs.

(a and b) To confirm the absence of contaminating viral RNA in HIV-naïve donor samples, HDACi monotherapies ,PKC agonist, and combinations of HDACi and PKC agonist, were evaluated using

culture supernatants via qRT-PCR. (**c and d**) Negative donor MDDC+CD4 T cell co-cultures were tested for IFN-γ production via ELISpot using various latency reversing conditions. (**e**) The presence of viral RNA was quantified using qRT-PCR in five donors (n=5) infected at the acute stage of infection. Assay cutoffs for ELISpot and qRT-PCR are 50 SFU/106 CD4 T cells and 30 viral copies/reaction. Results shown are group means +/- SEM. (**f**) To verify that the concentrations of stimuli used could cause latency reversal, we used the J-Lat cell line. The various stimuli were incubated overnight with the J-Lat cells and the numbers of GFP positive cells quantified by Flow cytometry for three independent experiments. (**g**) To determine the toxicity of the HDACi and PKC agonist drugs, live/dead staining was used to discriminate the populations and the percentage of GFP+ cells in drug titration studies plotted (100x - 0.01x). The blue arrow shows the concentration of HDACi and PKC agonist used.

Table 3-1: <u>Baseline characteristic of study volunteers.</u>

Volunteer	Age	Time between EDS and first positive test	Days before ART initiation after first positive test	Days on ART prior to PBMC donation	Days on ART until viral suppression	ART regimen since diagnosis	Baseline VL at PBMC donation	Baseline CD4 levels at PBMC donation	HIV-1 DNA frequency in CD4 T cells	Hep B/C Status
P1	33	61.5	34	748	125	Atripla	<20	710	1/400	Negative
P2	45	33.5	45	1206	159	Atripla	<20	684	1/9025	Negative
P3	47	77	22	809	231	Atripla	<20	1179	1/33	Negative
P4	34	6.5	34	1206	188	Atripla	<20	456	1/1324	Negative
P5	36	26	18	795	116	Atripla	<20	805	1/320	Negative
P6	31	207.5	20	685	139	Eviplera	<20	684	1/715	Negative
P7	41	102	54	618	65	Tenofovir, Emtricitabine, Darunavir	<20	561	1/29	Negative
P9	52	61	25	1033	107	Eviplera	<20	1220	1/16458	Negative
P8	25	46.5	40	855	268	Atripla	<20	1038	1/688	Negative
Average	38	69	32	884	155	N/A	<20	815	1/3221	Negative

[A]N.D. = Not Determined. DNA frequency could not be determined due to limited sample availability.
[B]Average HIV-1 DNA frequency was determined using the average of all samples for which a value could be determined (n=7). Samples labeled N.D. were excluded from this calculation.

Nine HIV-1 infected volunteers were enrolled in this study. Volunteers were identified through the SPARTAC (Short Pulse Anti-Retroviral Therapy at HIV Seroconversion) study and recruited through the HIV adult clinical services at St Mary's Hospital (Imperial College NHS trust). All study

participants provided written informed consent. All nine volunteers were recruited based on early HIV diagnosis and treatment with unabated cART-mediated suppression of plasma viremia (<50copies/ml HIV-1 RNA). The participants, age, date of HIV diagnosis, date of cART initiation, cART regimen, viral loads, CD4$^+$ T cell numbers, time of enrolment and HepB/C status were all recorded. The estimated date/time of seroconversion (EDS) was calculated as the midpoint between the most recent negative and the first positive test for patients.

Supplemental Table 1.

qRT-PCR	5' LTR PA	CCCACTGCTTAAGCCTCAATAAAGC
	GagR	CTTACTTTTGTTTTGCTCTTC
	TBP_F	TGCACAGGAGCCAAGAGTGAA
	TBP_R	CACATCACAGCTCCCCACCA
Illumina Env PCR	E80	CCAATTCCCATACATTATTGTG
	E125	CAATTTCTGGGTCCCCTCCTGAGG
	ED14	TCTTGCCTGGAGCTGTTTGATGCCCCAGAC
	EnvB	AGAAAGAGCAGAAGACAGTGGCAATGA
Illumina LTR PCR	5'LTRPA	CCCACTGCTTAAGCCTCAATAAAGC
	GagRev	TTCTAGCTCCCTGCTTGCCCATACTA
	MiS_PBSdt-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAAAAATCTCTAGCAGTGGCGCCCGAACAG
	MiS_GAGdt-R	GTCTCGTGGGCTCGGAGATGTGTATAAGTGACAGTTTCCAGCTCCCTGCTTGCCCATACTA

142

3.10 References

1. Arts, E. J. & Hazuda, D. J. HIV-1 antiretroviral drug therapy. *Cold Spring Harb. Perspect. Med.* (2012). doi:10.1101/cshperspect.a007161

2. McIlroy, D. Do HIV-specific CTL continue to have an antiviral function during antiretroviral therapy? If not, why not, and what can be done about it? *Front. Immunol.* (2013). doi:10.3389/fimmu.2013.00052

3. Chun, T. W. *et al.* Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc. Natl. Acad. Sci. U. S. A.* (1997). doi:10.1073/pnas.94.24.13193

4. Finzi, D. *et al.* Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science (80-.).* (1997). doi:10.1126/science.278.5341.1295

5. Viard, J. P. *et al.* Impact of 5 years of maximally successful highly active antiretroviral therapy on CD4 cell count and HIV-1 DNA level. *AIDS* (2004). doi:10.1097/00002030-200401020-00005

6. Finzi, D. *et al.* Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat. Med.* **5**, 512–517 (1999).

7. Siliciano, J. D. *et al.* Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. *Nat. Med.* **9**, 727–728 (2003).

8. Deeks, S. G. HIV: Shock and kill. *Nature* (2012). doi:10.1038/487439a

9. Pegu, A. *et al.* Activation and lysis of human CD4 cells latently infected with HIV-1. *Nat. Commun.* (2015). doi:10.1038/ncomms9447

10. Laird, G. M. *et al.* Ex vivo analysis identifies effective HIV-1 latency-reversing drug combinations. *J. Clin. Invest.* **125**, 1901–1912 (2015).

11. Bullen, C. K., Laird, G. M., Durand, C. M., Siliciano, J. D. & Siliciano, R. F. New ex vivo approaches distinguish effective and ineffective single agents for reversing HIV-1 latency in vivo. *Nat. Med.* **20**, 425–429 (2014).

12. Blazkova, J. *et al.* Effect of histone deacetylase inhibitors on HIV production in latently infected, resting CD4+ T cells from infected individuals receiving effective antiretroviral therapy. *J. Infect. Dis.* (2012). doi:10.1093/infdis/jis412

13. Jones, R. B. *et al.* Histone deacetylase inhibitors impair the elimination of hiv-infected cells by cytotoxic t-lymphocytes. *PLoS Pathog.* (2014). doi:10.1371/journal.ppat.1004287

14. Bruner, K. M. *et al.* Defective proviruses rapidly accumulate during acute HIV-1 infection. *Nat. Med.* (2016). doi:10.1038/nm.4156

15. Douek, D. C. *et al.* HIV preferentially infects HIV-specific CD4+ T cells. *Nature* **417**, 95–98 (2002).

16. Shete, A. *et al.* Short Communication: HIV Antigen-Specific Reactivation of HIV Infection from Cellular Reservoirs: Implications in the Settings of Therapeutic Vaccinations. *AIDS Res. Hum. Retroviruses* **28**, 835–843 (2012).

17. Deeks, S. G. & Walker, B. D. The immune response to AIDS virus infection: Good, bad, or both? *Journal of Clinical Investigation* (2004). doi:10.1172/JCI200421318

18. Brodin, J. *et al.* Establishment and stability of the latent HIV-1 DNA reservoir. *bioRxiv* (2016). doi:10.1101/053983

19. Abrahams, M. R. *et al.* The replication-competent HIV-1 latent reservoir is primarily established near the time of therapy initiation. *Sci. Transl. Med.* (2019). doi:10.1126/scitranslmed.aaw5589

20. Chun, T. W. *et al.* In vivo fate of HIV-1-infected T cells: Quantitative analysis of the transition to stable latency. *Nat. Med.* **1**, 1284–1290 (1995).

21. Poon, B., Grovit-Ferbas, K., Stewart, S. A. & Chen, I. S. Y. Cell cycle arrest by Vpr in HIV-1 virions and insensitivity to antiretroviral agents. *Science (80-.).* (1998). doi:10.1126/science.281.5374.266

22. Stewart, S. A., Poon, B., Song, J. Y. & Chen, I. S. Y. Human Immunodeficiency Virus Type 1 Vpr Induces Apoptosis through Caspase Activation. *J. Virol.* (2000). doi:10.1128/jvi.74.7.3105-3111.2000

23. Sengupta, S. & Siliciano, R. F. Targeting the Latent Reservoir for HIV-1. *Immunity* (2018). doi:10.1016/j.immuni.2018.04.030

24. Cicala, C. *et al.* HIV-1 envelope induces activation of caspase-3 and cleavage of focal adhesion kinase in primary human CD4+ T cells. *Proc. Natl. Acad. Sci. U. S. A.* (2000). doi:10.1073/pnas.97.3.1178

25. De Oliveira Pinto, L. M., Garcia, S., Lecoeur, H., Rapp, C. & Gougeon, M. L. Increased sensitivity of T lymphocytes to tumor necrosis factor receptor 1 (TNFR1)- and TNFR2-mediated apoptosis in HIV infection: Relation to expression of Bcl-2 and active caspase-8 and caspase-3. *Blood* (2002). doi:10.1182/blood.V99.5.1666

26. Mbonye, U. & Karn, J. Transcriptional control of HIV latency: Cellular signaling pathways, epigenetics, happenstance and the hope for a cure. *Virology* **454–455**, 328–339 (2014).

27. Pankrac, J. *et al.* A heterogeneous human immunodeficiency virus-like particle (VLP) formulation produced by a novel vector system. *npj Vaccines* **3**, 2 (2018).

28. Keele, B. F. *et al.* Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc. Natl. Acad. Sci. U. S. A.* (2008). doi:10.1073/pnas.0802203105

29. Günthard, H. F. *et al.* Effect of Influenza Vaccination on Viral Replication and Immune Response in Persons Infected with Human Immunodeficiency Virus Receiving Potent Antiretroviral Therapy. *J. Infect. Dis.* (2000). doi:10.1086/315260

30. Ho, D. D. HIV-1 viraemia and influenza. *The Lancet* (1992). doi:10.1016/0140-6736(92)91321-X

31. O'Brien, W. A. *et al.* Human immunodeficiency virus-type 1 replication can be increased in peripheral blood of seropositive patients after influenza vaccination. *Blood* (1995). doi:10.1182/blood.v86.3.1082.bloodjournal8631082

32. Ortigão-de-Sampaio, M. B. *et al.* Increase in plasma viral load after oral cholera immunization of HIV-infected subjects. *AIDS* (1998). doi:10.1097/00002030-199814000-00001

33. Staprans, S. I. *et al.* Activation of virus replication after vaccination of HIV-1-infected individuals. *J. Exp. Med.* (1995). doi:10.1084/jem.182.6.1727

34. Yek, C. *et al.* Standard vaccines increase HIV-1 transcription during antiretroviral therapy. *AIDS* (2016). doi:10.1097/QAD.0000000000001201

35. Lassen, K. G., Hebbeler, A. M., Bhattacharyya, D., Lobritz, M. A. & Greene, W. C. A flexible model of HIV-1 latency permitting evaluation of many primary CD4 T-cell reservoirs. *PLoS One* (2012). doi:10.1371/journal.pone.0030176

36. Spina, C. A. *et al.* An In-Depth Comparison of Latent HIV-1 Reactivation in Multiple Cell Model Systems and Resting CD4+ T Cells from Aviremic Patients. *PLoS Pathog.* (2013). doi:10.1371/journal.ppat.1003834

37. Hosmane, N. N. *et al.* Proliferation of latently infected CD4+ T cells carrying replication-competent HIV-1: Potential role in latent reservoir dynamics. *J. Exp. Med.* (2017). doi:10.1084/jem.20170193

38. Eriksson, S. *et al.* Comparative Analysis of Measures of Viral Reservoirs in HIV-1 Eradication Studies. *PLoS Pathog.* (2013). doi:10.1371/journal.ppat.1003174

39. Ho, Y. C. *et al.* XReplication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. *Cell* (2013). doi:10.1016/j.cell.2013.09.020

40. Procopio, F. A. *et al.* A Novel Assay to Measure the Magnitude of the Inducible Viral Reservoir in HIV-infected Individuals. *EBioMedicine* (2015). doi:10.1016/j.ebiom.2015.06.019

41. Kourjian, G. *et al.* HIV Protease Inhibitor–Induced Cathepsin Modulation Alters Antigen Processing and Cross-Presentation. *J. Immunol.* (2016). doi:10.4049/jimmunol.1600055

42. Kourjian, G. *et al.* Sequence-Specific Alterations of Epitope Production by HIV Protease Inhibitors. *J. Immunol.* (2014). doi:10.4049/jimmunol.1302805

43. Kumar, A. *et al.* Limited HIV-1 Reactivation in Resting CD4 + T cells from Aviremic Patients under Protease Inhibitors. *Sci. Rep.* (2016). doi:10.1038/srep38313

44. Steinman, R. M., Mellman, I. S., Muller, W. A. & Cohn, Z. A. Endocytosis and the recycling of plasma membrane. *Journal of Cell Biology* (1983). doi:10.1083/jcb.96.1.1

45. Schade, A. E. *et al.* Dasatinib, a small-molecule protein tyrosine kinase inhibitor, inhibits T-cell activation and proliferation. *Blood* (2008). doi:10.1182/blood-2007-04-084814

46. Hütter, G. *et al.* Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N. Engl. J. Med.* **360**, 692–8 (2009).

47. Allers, K. *et al.* Evidence for the cure of HIV infection by CCR5Δ32/Δ32 stem cell transplantation. *Blood* (2011). doi:10.1182/blood-2010-09-309591

48. Gupta, R. K. *et al.* HIV-1 remission following CCR5Δ32/Δ32 haematopoietic stem-cell transplantation. *Nature* (2019). doi:10.1038/s41586-019-1027-4

49. Archin, N. M. *et al.* Expression of Latent HIV Induced by the Potent HDAC Inhibitor Suberoylanilide Hydroxamic Acid. *AIDS Res. Hum. Retroviruses* **25**, 207–212 (2009).

50. Darcis, G. *et al.* An In-Depth Comparison of Latency-Reversing Agent Combinations in Various In Vitro and Ex Vivo HIV-1 Latency Models Identified Bryostatin-1+JQ1 and Ingenol-B+JQ1 to Potently Reactivate Viral Gene Expression. *PLoS Pathog.* **11**, (2015).

51. Mohammadi, P. *et al.* Dynamics of HIV Latency and Reactivation in a Primary CD4+ T Cell Model. *PLoS Pathog.* **10**, (2014).

52. Shan, L. *et al.* Stimulation of HIV-1-Specific Cytolytic T Lymphocytes Facilitates Elimination of Latent Viral Reservoir after Virus Reactivation. *Immunity* (2012). doi:10.1016/j.immuni.2012.01.014

53. Baxter, A. E. *et al.* Single-Cell Characterization of Viral Translation-Competent Reservoirs in HIV-Infected Individuals. *Cell Host Microbe* (2016). doi:10.1016/j.chom.2016.07.015

54. Bruner, K. M. *et al.* A quantitative approach for measuring the reservoir of latent HIV-1 proviruses. *Nature* (2019). doi:10.1038/s41586-019-0898-8

55. Macatangay, B. J. C. *et al.* Therapeutic Vaccination with Dendritic Cells Loaded with Autologous HIV Type 1-Infected Apoptotic Cells. *J. Infect. Dis.* (2016). doi:10.1093/infdis/jiv582

56. Jacobson, J. M. *et al.* Dendritic cell immunotherapy for HIV-1 infection using autologous HIV-1 RNA: A randomized, double-blind, placebo-controlled clinical trial. *J. Acquir. Immune Defic. Syndr.* (2016). doi:10.1097/QAI.0000000000000926

57. Gay, C. L. *et al.* Immunogenicity of AGS-004 Dendritic Cell Therapy in Patients Treated during Acute HIV Infection. *AIDS Res. Hum. Retroviruses* (2018). doi:10.1089/aid.2017.0071

58. Lu, W., Wu, X., Lu, Y., Guo, W. & Andrieu, J. M. Therapeutic dendritic-cell vaccine for simian aids. *Nat. Med.* (2003). doi:10.1038/nm806

59. Gandhi, R. T. *et al.* A randomized therapeutic vaccine trial of canarypox-HIV-pulsed dendritic cells vs. canarypox-HIV alone in HIV-1-infected patients on antiretroviral therapy. *Vaccine* (2009). doi:10.1016/j.vaccine.2009.05.016

Title: Potent reactivation of latent provirus using a heterogenous virus-like particle formulation in individuals treated during chronic stage infection.

4.1 Introduction

HIV-1, the causative agent behind AIDS-related illnesses, has remained a global health concern for nearly four decades. During this time, the advent of antiretroviral therapy (ART) has drastically reduced HIV-associated mortality by abrogating viral replication and facilitating immune system recovery.[1] Unfortunately, ART remains non-curative due to the establishment of stably integrated proviral populations, or reservoirs, within host memory CD4 T cells. In the presence of ART, these reservoirs can persist indefinitely due to homeostatic proliferation in the absence of viral transcription, viral protein production, and/or virion formation.[2,3] This quiescent pool is established within the first few days following primary infection.[4] Early ART administration is insufficient to protect against the establishment of latent HIV-1 provirus, although it may contribute to a decrease in the overall reservoir size.[5,6] Because of the inability to prevent reservoir establishment with currently available therapeutics, many strategies have aimed to eliminate the proviral pool.

The 'shock-and-kill' strategy for HIV-1 eradication aims to induce transcriptional reactivation of latent provirus so that infected cells may be removed via immune-mediated clearance or viral cytopathic effects.[7,8] During this time, cART is maintained to prevent *de novo* infection of bystander cell populations by induced HIV-1 virions. Several latency reversal agents (LRAs) have exhibited the ability to re-active transcriptionally silent proviral reservoirs. Histone deacetylase inhibitors (HDACi) are canonical LRAs capable of enabling reactivation through deacetylation of histones involved in chromatin condensation, thus creating a euchromatic environment.[9,10] Such strategies exhibit good tolerance *in vivo*, but largely fail to reduce the overall reservoir size.[11,12] Combinational therapies including HDACi and PKC agonists may further potentiate the effect of these latency reversing agents (LRA).[13] Alternatively, there have been an increasing number of studies that achieve *in vitro* latency reversal by targeting innate immune sensing molecules, such as toll-like receptors (TLR).[14–17] Despite their modest effects, TLR agonists largely lack specificity and require additional investigation. Due to the poor therapeutic index of

148

currently available LRAs, there is an increasing need to discover novel avenues for potent induction of proviral transcription.

The primary challenge with latency reversal is creating a novel therapeutic 'shock' that is both efficacious and well tolerated. This proves difficult as the predominant reservoir is thought to persist in HIV-specific CD4 T cells.[18,19] Moreover, only a fraction of CD4 T cells harbour replication competent latent provirus, with approximately 97.6% of proviruses containing fatal mutations or deletions.[20] More studies have recently sought to investigate the potential of DC-mediated therapies to induce cell transcriptional reactivation, due to their low toxicity and high specificity.[21] However, such approaches often fail to account for the viral quasi-species and therefore, MHC-II:peptide complexes may not sufficiently engage with all the HIV-specific TCRs present within the host. This becomes especially difficult with individuals diagnosed and treated during the chronic stage of infection, as this population will exhibit more diversity within their proviral reservoir. Despite this, evidence suggests that the replication competent viral reservoir is seeded around the time of cART initiation.[22] With this in mind, we proposed that creating a virus-like particle formulation that encompasses the entire host quasi-species immediately prior to cART initiation would potently induce latency reversal within the replication-competent proviral reservoir. Previously, we have evaluated our activator vector (ACT-VEC) in donors diagnosed and treated during acute infection (refer to chapter 3).[23] Our findings show that ACT-VEC potently stimulates transcriptional reactivation of CD4 T cell-specific reservoir to a greater extent than clinically relevant HDACi and PKC agonist monotherapies. Now, we aim to evaluate ACT-VEC in donors diagnosed and treated during chronic infection, which represent the vast majority of individuals currently infected with HIV-1, today.

4.2 Materials and Methods:

4.2.1 Experimental Design

Our <u>act</u>ivator <u>vec</u>tor(ACT-VEC) formulation was created to potently induce cell transcriptional reactivation as part of a therapeutic HIV-1 curative strategy. These studies are based upon literature suggesting that the predominant latent reservoir is established within HIV-specific CD4 T cells.[18] On this finding, we hypothesized that presenting peptides representative of a diverse HIV-1 pool could engage with the maximal number of HIV-specific TCRs on latently infected cells. To accomplish this, we developed a diverse virus-like particle (VLP) formulation representative of the viral quasi-species from several chronically infected, subtype D individuals. Particles were made using plasma from volunteers immediately prior to cART initiation. The objectives of this study are based on a previous study that revealed ACT-VEC was a potent LRA in donors identified and treated during acute infection. ACT-VEC will now be evaluated in the context of donors diagnosed and treated during chronic infection. To evaluate this, we will evaluate the latency reversing potential of ACT-VEC relative to alternative latency reversing regimens, such as: PKC agonists, HDACis, and CD3/CD28 agoists. Moreover, we aim identify if we can decrease immunogenicity through highly-specific targeting of HIV-specific T cells.

4.2.2 Production of Virus-Like Particle Formulation:

HIV-positive serum from subtype D donors was collected immediately prior to cART initiation for the preparation of virus-like particle formulations. Samples were collected during the chronic stage of infection. Viral RNA was extracted from cells using a Qiagen viral RNA extraction kit (Qiagen, USA) and reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Reverse transcription utilized specific primers to create two cDNA fragments which, when combined, fully encompass the full length of HIV-1 genome. cDNA Fragments were subjected to a nested PCR protocol to create overlapping 5' and 3' fragments. A list of primers can be found in **Table S1**. Primers contained an Integrase (IN) mutation which generate an AAH>RRK substitution, rendering the integrase non-functional.[23] The 5' primer also contained mutations within the ψ packaging region (698C>T, 718C>G, 719G>T, 720G>C, 721C>G, 722AT, 723A>T, 724G>C, and 731G>A, referred to as dS.1), thus rendering the particles incapable of encapsidating viral genomic material. The

resulting genomic fragments were transfected into *Saccharomyces cerevisiae* at an equivalent ratio and with 2μg of SacII linearized pRECΔgag-U3/URA3 plasmid. The transformation yields triple-recombinant yeast colonies that replace U3/URA3 with a donor-derived near-full-length viral genome (pRECΔgag-U3/URA3 →pREC_nfl_SubD.dS1.IN$_{AAH>RRK}$). Selection of pREC_nfl_SubD.dS1.IN$_{AAH>RRK}$ occurs on complete media lacking leucine (C-Leu) plates containing fluoroorotic acid (FOA). This vector formulation is devoid of a 5' LTR, cannot encapsidate gRNA (due to the dS.1 mutation), and does not contain a functional integrase gene. Yeast mini-prep was performed on colonies to isolate plasmids prior to transformation into bacteria. Plasmids were purified using a MaxiPrep (Qiagen, USA) and transfected into Human embryonic kidney (HEK) 293T cells using Fugene-6 (Promega, USA) by following the manufacturer-provided protocols. VLPs resulting from transfection were centrifuged at 300g for 10 minutes to remove any potentially contaminating cells. Cell-free supernatant containing VLPs were then run through a 100 KDa MWCO centrifuge tube (Amicon, USA) before ultracentrifugation at 40,000g. Pelleted VLPs were re-suspended in sterile PBS containing 2% heat-inactivated FBS. Verification of viral particles included, i) size and polydispersity analysis (dynamic light scattering), ii) p24 production, iii) packaging knockdown, and iv) RT activity. After all of the aforementioned steps are performed, virus-like particles are referred to as ACT-VEC.

4.2.3 Isolation and culture of donor CD4 T lymphocytes and monocyte-derived dendritic cells (MDDCs):

Peripheral blood mononuclear cells (PBMCs) were isolated from HIV-infected donors via density gradient centrifugation. For T cell purification, PBMCs were treated with magnetically-labeled microbeads to negatively select for CD4 T lymphocytes (CD4 T cell isolation kit, Miltenyi, Biotec, USA) as-per manufacturer instructions. Isolated cells are immediately used after purification. Purity of CD4 T cell isolations has been described elsewhere (refer to chapter 3).

Monocyte-derived dendritic cells (MDDCs) were purified from PBMCs via 2-hour plastic adherence in a T-75 culture flask (Sarstedt, Ca) at 37°C, 5% CO$_2$. After two hours, all media (RPMI; 10% FCS, 2mM L-Glutamine) containing non-adherent cells was removed and added to a separate T-75 flask. The adherent monocyte population received IL-4 (500 U/mL) and GM-CSF (1000 U/mL) for the duration of the 6-day maturation process to produce MDDCs. Halfway

through the maturation process (day 3), ½ of media was removed and centrifuged at 300g for 10 minutes. Media was discarded, ensuring the cell pellet it uninterrupted, prior to replenishment with fresh media containing IL-4 and GM-CSF.

4.2.4 IFN-γ ELISpot Assay

Human IFN-γ enzyme-linked immunosorbent spot (ELISpot) assays (Mabtech, USA) were performed on MDDC+T cell co-cultures. Performance of the ELISpot assay abided by manufacturer's protocol. Briefly, ELISpot plates are washed 5x with sterile PBS prior to being blocked with RPMI (10% FBS, 2mM L-Glutamine) for 30 minutes. Plates were washed 3x with sterile PBS before the addition of cells at a concentration of $1x10^6$ CD4 T cells/mL. Plates containing stimulation were incubated for 16 hours. After incubation, culture supernatants were gently aspirated and removed for additional experiments. Wells were washed 3x with sterile PBS prior to addition of biotinylated anti-IFN-γ antibody (1 µg/mL) for 2 hours. After this time, plates were washed and streptavidin-HRP was added for 1 hour. Plates were washed and subjected to 100 µl/well TMB peroxidase (Millipore Sigma, USA) for up to 15 minutes. Plates dried for 24 hours prior to imaging and counting of spots.

4.2.5 Latency Reversing Co-Culture Assay

Briefly, MDDCs were pulsed on day 6 post-plastic adherence with, i) media, ii) 5 µg/mL (p24) of RT-equivalent ACT-VEC (subtype A,B, or D), iii) CMV-tetanus-flu (CTF) recall antigen [250 ng/peptide/mL Flu M2 protein (bei Resources, USA), 2 µg/mL Tetanus toxoid (Statens Serum Institut, DK), 5 µg/mL pp65 HCMV peptide pool (NIH AIDS Reagent Program, USA)], or iv) or pooled malarial antigen. Fresh, autologous CD4-purified T cells were co-cultured with antigen-pulsed MDDCs on day 7 following plastic adherence. Cells were co-cultured at a ratio of 4 T cells to 1 DC at a concentration of $1x10^6$ CD4 T cells/mL (ie., $2.5x10^5$ MDDC/mL). For conditions that did not require MDDC-pulsing (PMA/Ionomycin, αCD3/αCD28, HDACi), stimuli were added on day 7 directly to unpulsed MDDC-T cell co-culture at concentrations described previously (refer to chapter 3). All latency reversing agents were used at concentrations previously outlined within the literature. Enfurvitide (T20; 10 µM) was added to all co-cultures to prevent infection of bystander cell populations upon reactivation. Co-cultures were prepared in dilution plates before transfer and 16h incubation on IFN-γ ELISpot plates, as described previously. Upon

completion of ELISpot, co-cultured cells are removed from the ELISpot plates and added to a round bottom 96-well plate for a total length of 72 hours.

4.2.6 Quantification of cell-free supernatant HIV-1 mRNA

After a 72-hour total culture, plates were spun at 300g for 10 minutes and 0.2mL of cell-free supernatant was collected/well, which represents $2x10^5$ CD4 T cells. Viral RNA is extracted from cell supernatant using a viral RNA isolation kit (Qiagen, USA). cDNA was synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystems, Ca). Quantitative reverse transcription PCR (qRT-PCR) was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems, Ca) on QuantStudio5 (Applied Biosystems, Ca). Briefly, 1μL of cDNA was diluted in 19 μL of sterile H_2O and added to 30 μL of TaqMan Fast Advanced Master mix as per the manufacturer instructions. Primers and probes can be found in **Table S2**. The standard curve was derived from 1/3 dilutions of XbaI-digested pREC_5'LTR-SBF-1 plasmid with a known concentration. For each dilution, 20 μl of standard is mixed with 30 μl of TaqMan master mix. The cycling conditions were as follows: 60°C for 2 minutes (1x), 95°C for 20 seconds (1x), 95°C for 1 second (40 cycles), 60°C for 43 seconds (40 cycles). Results were analyzed using GraphPad Prism (GraphPad Software, USA).

4.2.7 Statistical Analysis:

Herein, $P < 0.05$ was used as a threshold for statistical significance. Mann-Whitney non-parametric T test or Wilcoxon matched pairs signed rank tests were used to test for intra- and intersample significance, as indicated throughout. Correlative data was interpreted using a two-tailed Spearman Rank Correlation. Only donors who exhibited sustained suppression of viral loads (>6 months before sample donation) were enrolled in this study. No blinding was performed in this study as all samples were treated identically throughout testing.

4.3 Results

4.3.1 ACT-VEC potently induces latency reversal during chronic stage infection.

As most individuals are diagnosed and cART-treated during the chronic stage of infection, there exists a need for novel therapeutics capable of potently inducing latency reversal at this stage of infection. During primary infection, activated cells may undergo death via caspase-1/3-mediated events, die from viral cytopathic effects or, alternatively, transition to a memory phenotype that facilitates cell survival.[7,24–26] The latent reservoir is primarily established in CD4 T cells with HIV-specific TCRs, as these cells respond to infection and therefore make ideal, permissive targets.[18,19] More recent studies have confirmed that antigen-specific clones contribute to the latent reservoir and further suggest that repeat exposure to recall antigens contribute to reservoir persistence.[27] The timing of cART initiation also provides insight, as majority of the replication competent reservoir is established during this period.[22] Such findings suggest that the creation of a heterogenous virus-like particle (VLP) formulation that encompasses the viral quasi-species immediately prior to cART initiation may optimally stimulate latency reversal of HIV-specific CD4 T cells.[28]

The <u>act</u>ivator <u>vec</u>tor (ACT-VEC) formulations presented herein are based on previous findings that ACT-VEC can potently induce latency reversal at the acute stage of infection (Mann et al., 2020; manuscript accepted). ACT-VEC contains all viral proteins, is morphologically identical to wildtype HIV-1, and lacks viral genomic material thus rendering it non-infectious.[28] In this study, our VLP formulation was developed using HIV-positive sera from chronically-infected, subtype D donors (referred to as ACT-D). Plasma was collected from donors immediately prior to cART initiation, as VLPs derived during this period may optimally stimulate CD4 T cells harboring replication competent provirus.

The ability of ACT-D to induce latency reversal was evaluated using PBMCs from cART-treated, chronically-infected, subtype D donors (ChrD-cART). Monocyte-derived dendritic cells (MDDC) from ChrD-cART individuals (n=4) were pulsed with ACT-D (5 μg/mL) and subsequently co-cultured with autologous CD4 T cells ($1x10^6$ cells/mL). Culture supernatants were harvested after 3 days post-stimulation to detect viral 5' LTR RNA via qRT-PCR. All stimulation conditions received the HIV-1 fusion inhibitor, enfurvitide (T20; 10 μM) to protect bystander cells from *de*

novo infection upon reactivation. Across the enrolled study participants, ACT-D induced an average 6.70- and 26.47-fold increase in detectable culture supernatant RNA relative to the global T cell activators, PMA/Ionomycin and αCD3/αCD28, respectively (Figure 4-1 a,b). Notably, latency reversal was achieved in all donors (4/4) treated with ACT-D-pulsed MDDC, compared to 50% (2/4) and 75% (3/4) of PBMCs treated with PMA/Ionomycin or αCD3/αCD28. Interestingly, latency reversal was comparable between ACT-D and ACT-B, despite the latter preparation being from a different subtype. This finding may suggest that there is a large degree of overlap in the TCR specificity of CD4 T cells responding to subtype D and B infections. Moreover, ACT-VEC therapies, on average, were better inducers of HIV RNA production relative to non-HIV-1 antigen cocktails, such as malarial pool antigen or CMV/tetanus/flu (CTF). Significance could not be ascertained due to the limited number of donors.

To determine the antigenicity of the various latency reversing agents, MDDC-T cell co-cultures were performed in 96-well IFN-γ ELISpot plates. MDDC were cultured with T cells at a ratio of 1:4, wherein T cells were seeded at a concentration of 1×10^6 cells/mL. Despite heightened viral RNA production, ACT-VEC formulations maintain low antigenicity relative to other T cell activators, including alternative peptide:MDDC formulations (malaria, CTF) (Figure 4-1 c,d). All non-ACT-VEC therapies were significantly more antigenic than the media control ($p < 0.05$), whereas ACT-D ($p = 0.4857$) and ACT-B ($p = 0.4286$) did not differ significantly. The ratio of induced viral copies to IFN-γ SFUs indicates the relative amount of RNA release per activated cell (Figure 4-1e). If the reservoir has high specificity for a specific subset of memory cells (ie. HIV-specific memory CD4 T cells), the ratio should be highest for that condition. With this in mind, it is evident that ACT-VEC therapies exhibit higher ratios than alternative regimens.

As mentioned in chapter 3, it is important to note that culture supernatant RNA was detected using primers specific for the 5' LTR (R-U5) of HIV-1. As this region is absent within ACT-VEC formulations, qRT-PCR will only detect the presence of induced virus and not ACT-VEC. The inability to detect ACT-VEC VLPs using our qRT-PCR has been detailed elsewhere (refer to chapter 3).

Herein, we identify that latency reversal can be achieved in ChrD-cART donors using inter- and intra-subtype ACT-VEC formulations. Based on the assumption that a majority of latent provirus resides within the HIV-specific CD4 T cell population, it is possible to hypothesize that the similar latency-reversing potential of ACT-B and ACT-D is due to a high degree of similarity in TCR specificity across subtypes. However, it is important to note that latency reversal is also achieved with malarial antigens and CTF. This cohort has likely encountered malaria, CMV, flu, or tetanus at some point during their life as a result of natural exposure or vaccination. Based on this, it is possible that CD4 T cells with alternative TCR specificities were active during the period of untreated HIV infection, thus facilitating latency establishment in alternative CD4 T cell reservoirs. Despite this, a majority of viral reactivation is achieved in the ACT-treated conditions. Moreover, stimulation with global T cell activators were less efficient than any MDDC-pulsed antigen conditions. This finding is in line with other studies indicating that latency reversal is best achieved in the context of TCR stimulation.[21,29,30]
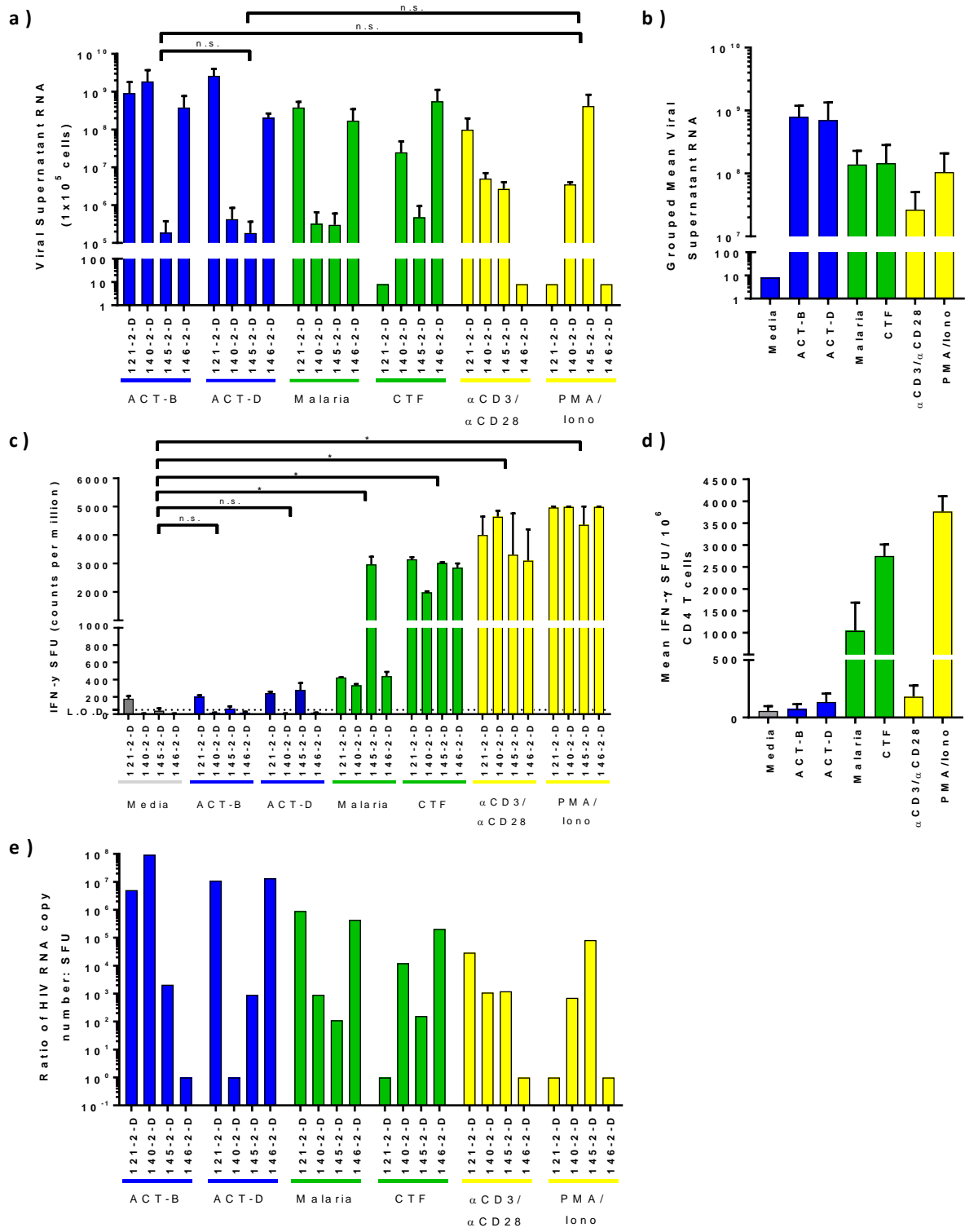
Figure 4-1: ACT-VEC potently induces latency reversal in chronically-infected, patient-derived samples.

cART-treated donors (n=4) diagnosed and treated during chronic infection that exhibited viral suppression (<50 copies/mL) were enlisted in this study. All studies in this cohort are performed on subtype D individuals. ACT-B (5 µg/mL) and ACT-D (5 µg/mL) were inter- and intra-subtype specific stimuli used to pulse autologous MDDCs. Malaria (1 µg/mL) and CTF (CMV, Tetanus toxoid, Flu M1) were used as non-HIV-specific stimuli for MDDC pulsing. αCD3/αCD28 and PMA/Ionomycin served as assay positive controls and media served as a negative. **(a)** qRT-PCR of RNA collected from viral supernatant 3 days after co-culture. qRT-PCR values are background subtracted. **(b)** Grouped means were calculated for each of the co-culture conditions. **(c)** MDDCs were pulsed with any of ACT-B, ACT-D, malarial antigen, or CTF and subsequently co-cultured with autologous CD4 T cells. PMA/Ionomycin and αCD3/αCD28 serve as assay controls. Antigenicity of stimuli on CD4 T cells was calculated by measuring IFN-γ spot forming units (SFUs). **(d)** Averages of IFN-γ SFUs for each stimulus were calculated. **(e)** The amount of detectable HIV-1 culture supernatant RNA was compared to the antigenicity of each stimuli as a ratio (copy number:SFU). Grouped samples were used to determine mean effect (+- SEM) of the stimulations. Statistical significance was determined using the Mann-Whitney unpaired T test.

4.3.2 ACT-VEC induces latency to a similar or greater degree than HDACi, TLR agonist, and PKC agonist monotherapies.

Through evaluation of MDDC-pulsed latency reversal strategies, we have identified that ACT-VEC formulations are potent reactivators of proviral transcription using ChrD-cART PBMCs. We now seek to compare the potency of our ACT-VEC-mediated latency reversing response to clinically relevant LRAs which have been the focus of many clinical and pre-clinical trials.[13,31,32] For each latency reversing agent, dosing was first performed in the latently infected Jurkat cell line, J-Lat 6.3, as described previously (refer to chapter 3). Unpulsed, ChrD-cART-derived MDDC (n=4) was co-cultured with autologous CD4 T cells (1×10^6 CD4 T cell/mL) in the presence of the various monotherapies. Culture supernatants were collected after 3 days of cell culture and analyzed for viral RNA via qRT-PCR. Through stimulation with histone deacetylase inhibitors, latency reversal was consistently detected in 2/4 individuals (Figure 4-2 a,b). Alternatively, TLR 7&8 monotherapies, such as ssRNA, Imiquimod, or GS-9620 displayed a lesser ability to induce detectable RNA in culture supernatants. Although significance could not be obtained due to small sample size, it is important to note that, in contrast to the selected monotherapies, all ACT-pulsed MDDC co-cultures exhibited latency reversal.

Despite their variable ability to induce transcriptional reactivation, most monotherapies except for Bryostatin ($p<0.05$) maintained similarly low levels of IFN-γ production (Figure 4-3c). This is in-line with previous findings indicating that these regimens are well-tolerated *in vivo*.[33–36] However, when considering each monotherapy in the context of latency reversal, we can conclude that ACT-VEC formulations are *at least* as potent, if not more. This may be due to potent induction of T cells stimulated through the TCR, as opposed to alternative mechanisms of reactivation.
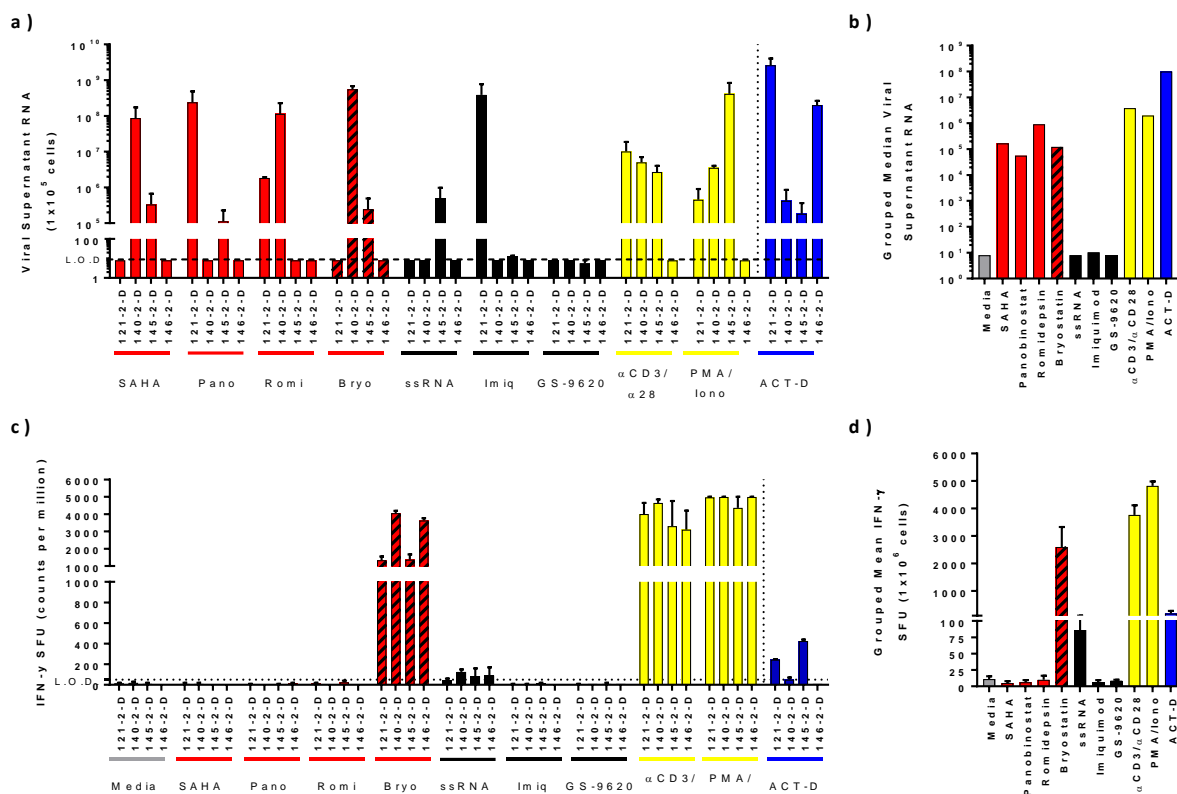
Figure 4-2: ACT-VEC can potently induce latency reversal to a similar or greater degree than other latency reversing agents.

ACT-VEC-pulsed MDDC were co-cultured with autologous CD4 T cells from HIV infected donors (n=4). Comparisons were made to clinically relevant latency reversing agents (LRA; HDACi, TLR agonist, PKC agonist) and T cell mitogens, including: SAHA, Panobinostat, Romidepsin, Bryostatin, ssRNA, Imiquimod, GS-9620, αCD3/αCD28, and PMA/Ionomycin. **(a)** Viral supernatants were collected and evaluated for the presence of viral RNA via qRT-PCR after 3 days post-stimulation. **(b)** Group median of each stimulation condition from (a). **(c)** Antigenicity was determined using IFN-γ ELISpot after overnight stimulation with pulsed MDDC, LRA, or T cell mitogen. ELISpot conditions were performed in duplicate at 1×10^5 CD4 T cells/well. **(d)** Grouped mean antigenicity of each condition group, represented as spot forming units per million cells (+-SEM).

4.3.3 ACT-VEC induces comparably more latency reversal during chronic stage infection

Previous studies conducted by our group evaluated HIV-1 latency reversal in the context of an acutely-infected donor cohort (refer to chapter 3). Transcriptional reactivation of individuals diagnosed and treated during the acute stage of infection represent the 'ideal' model for latency reversal. Specifically, during acute infection there are fewer opportunities for mutations within the viral genome and, as a direct consequence, fewer variants capable of escaping immune-mediated clearance upon reactivation. Mutations also contribute to viral diversity and, therefore, increase the number of CD4 T cells that can detect HIV-derived viral antigens. During acute infection, there are also fewer opportunities for co-infection with opportunistic pathogens. Co-infection facilitates the seeding of alternative reservoirs due to activation of T cell subsets with non-HIV-specific TCRs. The chronic cohort may also experience heightened levels of T cell anergy following cellular activation, further complicating cure at later stages of disease.[37] Consequently, the requirements of a therapeutic vaccine candidate become increasingly vague as HIV-1 infection continues in the absence of cART.

To evaluate the differential effect of ACT-VEC-induced transcriptional reactivation during acute and chronic infection, culture supernatant qRT-PCR was compared (Figure 4-3). At the chronic stage of infection, ACT-VEC had an ~4 log increase in mean detectable supernatant RNA (****$p < 0.0005$). This observation may be attributed to several factors. Firstly, the proviral reservoir in chronically-infected donors is expected to be substantially larger than during acute infection due to the prolonged absence of cART. Despite this, the diversity present within ACT-VEC formulations will ensure activation of many HIV-specific TCRs, including mutational variants.
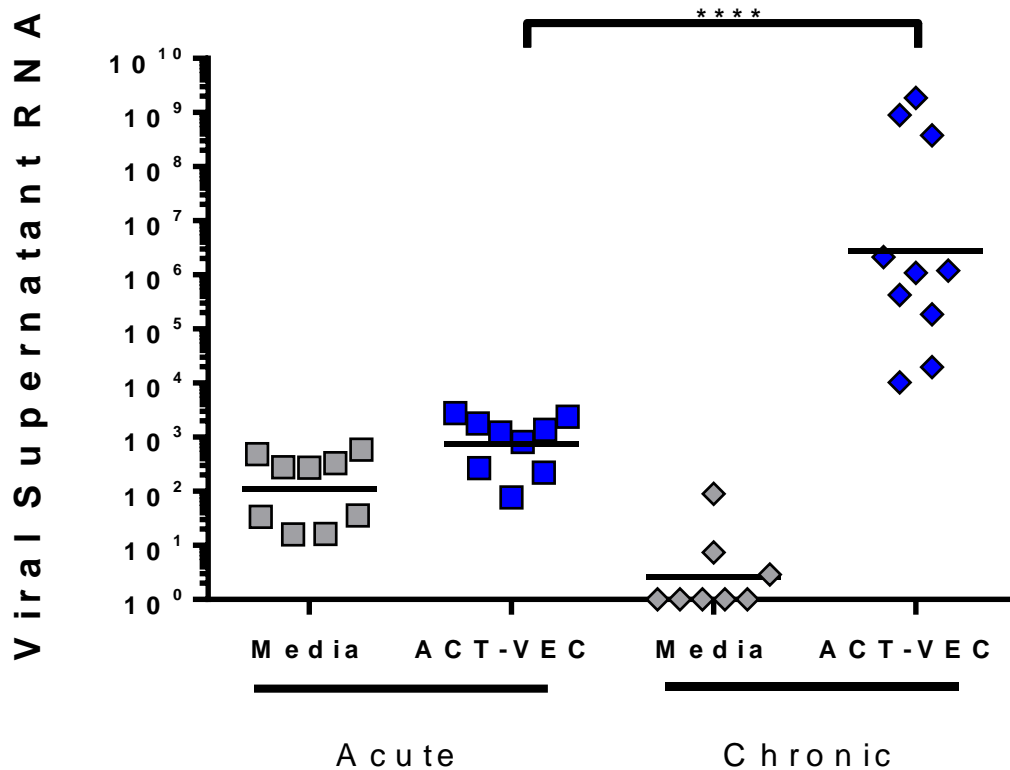
Figure 4-3: Comparison of ACT-VEC mediated latency reversal during the acute and chronic stages of infection.

Culture supernatants were collected 3 days after co-culture and evaluated for the presence of viral RNA using qRT-PCR. Donors from the acute (n=9) and chronic (n=10) stage of infection are represented as individual dots on the graph. A media control was used to determine the amount of background RNA detected in culture. Bars represent the median value within each population. Statistical analysis was performed using a Mann-Whitney unpaired T test (****p<0.005).

4.4 Discussion

Addressing the HIV-1 latent reservoir remains of the utmost importance for potential curative strategies. Latency is well-maintained over time due to several factors, including i) integration in sites of low transcriptional activity, ii) downregulation of host transcription factors, iii) through epigenetic modifications, and iv) through homeostatic proliferation.[29,38] Since the identification of the latent reservoir in the mid-90s, many different approaches have successfully induced transcriptional reactivation of the predominant CD4 T cell reservoir, albeit to varying degrees.[34–36,39,40] However, most LRAs fail to induce an immune-mediated 'kill' upon transcriptional reactivation, thus leading to a non-significant reduction in reservoir size. Contrarily, many of these latency-reversing strategies (such as HDACi) induce the production of pro-survival proteins, such as Bcl-2, and therefore prevent death via NK- and CD8-mediated cytotoxic responses.[41] *In vitro* studies of HDACi show that these drugs may directly inhibit proper functioning of cytotoxic cells.[42,43] Other regimens, such as PKC agonists, have shown a poor ability to reduce the reservoir size, *in vivo*.[35] Furthermore, these LRAs function through non-specific activation of T cells and may therefore heighten immune inflammation. The shortcomings of relevant LRA-based strategies suggest that there is a need for novel alternatives.

The complications associated with latency reversal are exacerbated during chronic-stage infection. Specifically, the longer HIV infected individuals remain untreated, the larger and more genetically diverse their latent proviral reservoirs become. Compared to acute infection, individuals diagnosed during chronic infection experience more opportunities for antigenic exposure to non-HIV peptides. In the absence of ART, exposure to these alternative peptides can contribute to seeding within non-HIV-specific T cell subsets. Furthermore, CD4 T cells isolated from the chronic stage of infection have previously been shown to exhibit heightened levels of anergy or dysfunction as a result of chronic immune activation.[37,44] As most individuals are diagnosed and cART-treated during chronic infection, this stage represents the primary focus of the curative vaccine research published to date.

At the acute stage of infection, we have previously shown that ACT-VEC induces transcriptional reactivation to a greater extent than HDACi and PKC agonist monotherapies (refer to chapter

3). This model is idealized, as early cART initiation limits reservoir establishment and, additionally, reduces the degree of viral diversity in seeded reservoirs.[45] Importantly, this study is in-line with our previous study and with existing literature which suggests that HIV provirus has a substantial reservoir in T cells with HIV-specific TCRs.[18,19] Based on these findings, we evaluated our inter- (ACT-B) and intra- (ACT-D) subtype virus-like particle formulations in the context of individuals diagnosed and cART-treated during the chronic stage of infection. This would expand on our data from the acute stage of HIV infection while also enabling us to determine whether a clade-specific or heterologous clade-based ACT-VEC would be more efficient at causing HIV latency reversal. We hypothesized that the optimal induction of latency reversal would require a highly matched, heterogenous, intra-subtype VLP formulation created using sera from HIV-infected individuals immediately prior to cART initiation. To that end, we stimulated clade D CD4 T cells with HIV-specific TCRs using clade B and D ACT-VEC pulsed MDDCs. We believed that ACT-D would outperform ACT-B a inducing latency reversal from ChrD-cART CD4 T cells due to the high amount of inter-subtype variability – approximately 25-35%.[46] Owing to this variability, ACT-B should lack many of the HIV-specific peptides required to induce transcriptional reactivation within ChrD-cART donors. Surprisingly, ACT-B and ACT-D induced comparable levels of latency reversal (Figure 4-1 a,b). This result was contrary to our hypothesis and may suggest that HIV-specific TCRs preferentially recognize immunodominant peptides that are present within both VLP formulations. Interestingly, similar responses cannot be achieved using pooled HIV-peptides (data not shown). Reasons for this could be, i) poor uptake or presentation of peptides, ii) poor peptide stability, or iii) a lack of diversity. Determining donor HLA type would help address this phenomenon, although this information is not available for the donors presented herein. Additionally, this could be addressed by identifying which peptides are presented by the donors' APCs simply by eluting them from MHC and detecting by Mass Spectrometry. These peptides could then be evaluated against known HIV immunodominant peptides. The matching immunodominant peptides could then be evaluated in latency reversal studies in clade B and D patient samples.

In this chapter, ACT-VEC clade D formulations were compared to clinically relevant LRAs to determine the efficacy of each approach. Because the function of HDACi and PKC agonists are

targeted towards CD4 T cells, these stimulations received un-pulsed MDDCs during co-culture. Un-pulsed MDDC-T cell co-culture, alone, was unable to induce latency reversal, as we were unable to detect viral budding through analysis of viral RNA in culture supernatants. Alternatively, HDACi and PKC agonists were able to induce latency reversal, albeit in a smaller proportion of donors relative to ACT-VEC. Interestingly, viral RNA was readily detectable in culture supernatants from samples pulsed with CTF (CMV/Tetanus/Flu) and malarial antigens, respectively. This indicates that, within this chronic-stage cohort, alternative, non-HIV-specific CD4 T cell reservoirs may have become established in response to opportunistic infections in the absence of cART. To identify differences between these latency reversing strategies, deep gene sequencing will be performed on outgrowth virus collected from each condition. This data will be compared to proviral sequences information and quantitative viral outgrowth (qVOA) data. Moreover, next generation technologies, such as the FISH/Flow assay and the intact proviral DNA assay (IPDA), will provide additional insight into the efficacy of our 'shock-and-kill' approach.[47,48]

In summation, we demonstrate that inter- and intra-subtype ACT-VEC formulations efficiently reactivate quiescent HIV-1 provirus in a ChrD-cART infected cohort. This information further suggests that TCR-mediated latency reversal is in response to immunodominant peptides or HLA molecules, although this finding requires additional investigation. Our study finds that a majority of latent provirus resides in the HIV-specific reservoir, although opportunistic infections prior to cART initiation may contribute to alternative reservoir seeding. Continuation of this study will aim to increase the number of ChrD-cART samples available for testing, followed by pre-clinical, *in vivo* modeling. The cumulative data suggests that ACT-VEC is a potent latency reversing agent that merits further investigation.

4.5 Tables

Table 4-1S. List of primers for VLP construct development

	Primer Name	Directionality	Sequence
5' Nested Set	INTR2 [A,B,C]	3'	CAATCATCACCTGCCATCTGTTTTCCATAATCCCTGAT GATCTTTGCGTGTGCTGCTGGCACTACTTTTATGTC
	PRIMERF [A]	5'	GGCGGCGACTGGTGAGTACGCCAAAAATTTTGACTA GCGGAGGCTAGAAGGAGAGAGATGG
	PRIMER_ F.A [B]	5'	AGCGGCGACTGGTGAGTACGCCTTTTTTGACTAGCGG AGGCTAGAAGGAGAGAGATGG
	0776F [C]	5'	CTAGAAGGAGAGAGAGATGGGGTGCGAG
3' Nested Set	INTF2.A [D]	5'	ATACAGGATAATCAGGATATAAAGGTAGTGCCCGCA GCACATGCAAAGATCATTAGGGATTATGGAAAACAG ATG
	47M [D]	3'	TCCCTAGTTAGCCAGAGAGCTCCC

[A-D] Matching superscript indicates a specific primer set was used for the making of a VLP formulation in at least one donor sample. All preparations used the same 3' nested set (denoted '[D]').

166

Table 4-2S. List of probes and primers for qRT-PCR

Primer Name	Primer Type	Sequence
Sub AD Gag Probe	Probe	TCTGTTGTGTGACTCTGGTAACTAGAGATCCCTCAGACC
Sub AD 5' LTR PA	Forward Primer	CCCACTGCTTAAGCCTCAATAAAGC
Sub AD GagR	Reverse Primer	AGCTCCCTGCTTGCCCATACTA

*Concentrations are as follows: Probe = 100 nM, Primers Forward/Reverse = 600 nM.

4.6 References

1. Spivak, A. M. & Planelles, V. Novel Latency Reversal Agents for HIV-1 Cure. *Annu. Rev. Med.* (2018). doi:10.1146/annurev-med-052716-031710

2. Chomont, N. *et al.* HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. *Nat. Med.* (2009). doi:10.1038/nm.1972

3. Hosmane, N. N. *et al.* Proliferation of latently infected CD4+ T cells carrying replication-competent HIV-1: Potential role in latent reservoir dynamics. *J. Exp. Med.* (2017). doi:10.1084/jem.20170193

4. Whitney, J. B. *et al.* Rapid seeding of the viral reservoir prior to SIV viraemia in rhesus monkeys. *Nature* **512**, 74–77 (2014).

5. Strain, M. C. *et al.* Effect of Treatment, during Primary Infection, on Establishment and Clearance of Cellular Reservoirs of HIV-1. *J. Infect. Dis.* (2005). doi:10.1086/428777

6. Eriksson, S. *et al.* Comparative Analysis of Measures of Viral Reservoirs in HIV-1 Eradication Studies. *PLoS Pathog.* (2013). doi:10.1371/journal.ppat.1003174

7. Sengupta, S. & Siliciano, R. F. Targeting the Latent Reservoir for HIV-1. *Immunity* (2018). doi:10.1016/j.immuni.2018.04.030

8. Chun, T. W. *et al.* In vivo fate of HIV-1-infected T cells: Quantitative analysis of the transition to stable latency. *Nat. Med.* (1995). doi:10.1038/nm1295-1284

9. Jiang, G., Espeseth, A., Hazuda, D. J. & Margolis, D. M. c-Myc and Sp1 Contribute to Proviral Latency by Recruiting Histone Deacetylase 1 to the Human Immunodeficiency Virus Type 1 Promoter. *J. Virol.* (2007). doi:10.1128/jvi.01208-07

10. Verdin, E., Paras, P. & Van Lint, C. Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. *EMBO J.* (1993). doi:10.1002/j.1460-2075.1993.tb05994.x

11. Sagot-Lerolle, N. *et al.* Prolonged valproic acid treatment does not reduce the size of latent HIV reservoir. *AIDS* (2008). doi:10.1097/QAD.0b013e3282fd6ddc

12. Shirakawa, K., Chavez, L., Hakre, S., Calvanese, V. & Verdin, E. Reactivation of latent HIV by histone deacetylase inhibitors. *Trends in Microbiology* (2013). doi:10.1016/j.tim.2013.02.005

13. Laird, G. M. *et al.* Ex vivo analysis identifies effective HIV-1 latency-reversing drug combinations. *J. Clin. Invest.* **125**, 1901–1912 (2015).

14. Alvarez-Carbonell, D. *et al.* Toll-like receptor 3 activation selectively reverses HIV latency in microglial cells. *Retrovirology* (2017). doi:10.1186/s12977-017-0335-8

15. Thibault, S., Imbeault, M., Tardif, M. R. & Tremblay, M. J. TLR5 stimulation is sufficient to trigger reactivation of latent HIV-1 provirus in T lymphoid cells and activate virus gene expression in central memory CD4+ T cells. *Virology* (2009).

doi:10.1016/j.virol.2009.04.019

16. Novis, C. L. *et al.* Reactivation of latent HIV-1 in central memory CD4+ T cells through TLR-1/2 stimulation. *Retrovirology* (2013). doi:10.1186/1742-4690-10-119

17. Macedo, A. B. *et al.* Dual TLR2 and TLR7 agonists as HIV latency-reversing agents. *JCI insight* (2018). doi:10.1172/jci.insight.122673

18. Douek, D. C. *et al.* HIV preferentially infects HIV-specific CD4+ T cells. *Nature* **417**, 95–98 (2002).

19. Demoustier, A. *et al.* In patients on prolonged HAART, a significant pool of HIV infected CD4 T cells are HIV-specific. *AIDS* **16**, 1749–1754 (2002).

20. Bruner, K. M. *et al.* Defective proviruses rapidly accumulate during acute HIV-1 infection. *Nat. Med.* (2016). doi:10.1038/nm.4156

21. Kristoff, J., Rinaldo, C. R. & Mailliard, R. B. Role of dendritic cells in exposing latent HIV-1 for the kill. *Viruses* (2019). doi:10.3390/v12010037

22. Abrahams, M. R. *et al.* The replication-competent HIV-1 latent reservoir is primarily established near the time of therapy initiation. *Sci. Transl. Med.* (2019). doi:10.1126/scitranslmed.aaw5589

23. Pankrac, J. *et al.* A heterogeneous human immunodeficiency virus-like particle (VLP) formulation produced by a novel vector system. *npj Vaccines* **3**, 2 (2018).

24. Chun, T. W. *et al.* In vivo fate of HIV-1-infected T cells: Quantitative analysis of the transition to stable latency. *Nat. Med.* **1**, 1284–1290 (1995).

25. Stewart, S. A., Poon, B., Song, J. Y. & Chen, I. S. Y. Human Immunodeficiency Virus Type 1 Vpr Induces Apoptosis through Caspase Activation. *J. Virol.* (2000). doi:10.1128/jvi.74.7.3105-3111.2000

26. Cicala, C. *et al.* HIV-1 envelope induces activation of caspase-3 and cleavage of focal adhesion kinase in primary human CD4+ T cells. *Proc. Natl. Acad. Sci. U. S. A.* (2000). doi:10.1073/pnas.97.3.1178

27. Mendoza, P. *et al.* Antigen responsive CD4+ T cell clones contribute to the HIV-1 latent reservoir. *bioRxiv* 2020.01.10.902155 (2020). doi:10.1101/2020.01.10.902155

28. Pankrac, J. *et al.* A heterogeneous human immunodeficiency virus-like particle (VLP) formulation produced by a novel vector system. *npj Vaccines* **3**, (2018).

29. Mohammadi, P. *et al.* Dynamics of HIV Latency and Reactivation in a Primary CD4+ T Cell Model. *PLoS Pathog.* **10**, (2014).

30. van Montfort, T. *et al.* Dendritic cells potently purge latent HIV-1 beyond TCR-stimulation, activating the PI3K-Akt-mTOR pathway. *EBioMedicine* (2019). doi:10.1016/j.ebiom.2019.02.014

31. Bullen, C. K., Laird, G. M., Durand, C. M., Siliciano, J. D. & Siliciano, R. F. New ex vivo approaches distinguish effective and ineffective single agents for reversing HIV-1 latency in vivo. *Nat. Med.* **20**, 425–429 (2014).

32. Rasmussen, T. A. & Lewin, S. R. Shocking HIV out of hiding: Where are we with clinical trials of latency reversing agents? *Current Opinion in HIV and AIDS* (2016). doi:10.1097/COH.0000000000000279

33. Søgaard, O. S. *et al.* The Depsipeptide Romidepsin Reverses HIV-1 Latency In Vivo. *PLoS Pathog.* **11**, (2015).

34. Archin, N. M. *et al.* Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. *Nature* **487**, 482–485 (2012).

35. Gutiérrez, C. *et al.* Bryostatin-1 for latent virus reactivation in HIV-infected patients on antiretroviral therapy. *AIDS* **30**, 1385–92 (2016).

36. Archin, N. M. *et al.* Interval dosing with the HDAC inhibitor vorinostat effectively reverses HIV latency. *J. Clin. Invest.* (2017). doi:10.1172/JCI92684

37. Meyaard, L., Schuitemaker, H. & Miedema, F. T-cell dysfunction in HIV infection: anergy due to defective antigen-presenting cell function? *Immunol. Today* (1993). doi:10.1016/0167-5699(93)90279-T

38. Mbonye, U. & Karn, J. The Molecular Basis for Human Immunodeficiency Virus Latency. *Annu. Rev. Virol.* (2017). doi:10.1146/annurev-virology-101416-041646

39. Elliott, J. H. *et al.* Short-term administration of disulfiram for reversal of latent HIV infection: a phase 2 dose-escalation study. *Lancet HIV* (2015). doi:10.1016/S2352-3018(15)00226-X

40. Rasmussen, T. A. *et al.* Comparison of HDAC inhibitors in clinical development: effect on HIV production in latently infected cells and T-cell activation. *Hum. Vaccin. Immunother.* **9**, 993–1001 (2013).

41. Kim, Y., Anderson, J. L. & Lewin, S. R. Getting the "Kill" into "Shock and Kill": Strategies to Eliminate Latent HIV. *Cell Host and Microbe* (2018). doi:10.1016/j.chom.2017.12.004

42. Pace, M. *et al.* Histone Deacetylase Inhibitors Enhance CD4 T Cell Susceptibility to NK Cell Killing but Reduce NK Cell Function. *PLoS Pathog.* (2016). doi:10.1371/journal.ppat.1005782

43. Jones, R. B. *et al.* A Subset of Latency-Reversing Agents Expose HIV-Infected Resting CD4 + T-Cells to Recognition by Cytotoxic T-Lymphocytes. *PLoS Pathog.* (2016). doi:10.1371/journal.ppat.1005545

44. Sousa, A. E., Carneiro, J., Meier-Schellersheim, M., Grossman, Z. & Victorino, R. M. M. CD4 T Cell Depletion Is Linked Directly to Immune Activation in the Pathogenesis of HIV-1 and HIV-2 but Only Indirectly to the Viral Load. *J. Immunol.* (2002). doi:10.4049/jimmunol.169.6.3400

45.   Buzon, M. J. *et al.* Long-Term Antiretroviral Treatment Initiated at Primary HIV-1 Infection Affects the Size, Composition, and Decay Kinetics of the Reservoir of HIV-1-Infected CD4 T Cells. *J. Virol.* (2014). doi:10.1128/jvi.01046-14

46.   Taylor, B. S., Sobieszczyk, M. E., McCutchan, F. E. & Hammer, S. M. The challenge of HIV-1 subtype diversity. *New England Journal of Medicine* (2008). doi:10.1056/NEJMra0706737

47.   Bruner, K. M. *et al.* A quantitative approach for measuring the reservoir of latent HIV-1 proviruses. *Nature* (2019). doi:10.1038/s41586-019-0898-8

48.   Baxter, A. E. *et al.* Single-Cell Characterization of Viral Translation-Competent Reservoirs in HIV-Infected Individuals. *Cell Host Microbe* (2016). doi:10.1016/j.chom.2016.07.015

Chapter 5 – General Discussion

5.1 General Discussion

Despite ongoing efforts, the development of an efficacious vaccine for HIV-1 cure remains elusive. Therefore, in the absence of a cure, HIV will remain a global health concern, especially since 37.9 million individuals are currently living with HIV.[1] The advent of antiretroviral therapy has converted the HIV-related death sentence into a lifetime manageable chronic illness, and has been acknowledged by the World Health Organization as a cornerstone feature in efforts to stunt new infections and prolong life for affected individuals. The 90-90-90 initiative, which is based on the success of ART, aims for 90% of HIV-infected individuals to, i) know their HIV status, ii) receive ART, and iii) exhibit sustained viral suppression. While the original 2020 goal was not met, more people are aware of their seropositive status (~79%) and accessing treatment (~62%), worldwide.[1] Furthermore, death from AIDS-related causes has decreased by 33% between 2010-2019.[1] Unfortunately, this is not enough. If we compare the rate of new infections (1.7 million) and morality (770 000) for 2018, it becomes clear that the number of people burdened by infection is, in fact, rising. This suggests that HIV-1 is becoming increasingly onerous on economic and social frontiers. The UNAIDS estimates that $26.2 billion USD will be required for AIDS-response and drug-access initiatives, representing an increase of 38% since 2018, alone. Additionally, access and maintenance of antiretroviral therapy is not universally achievable. Specifically, low socioeconomic status is strongly associated with non-adherence to cART after initial diagnoses.[2] In this UK-based study, factors such as, sexual orientation, ethnic

origin, age, and financial instability all contributed to a rate of 32% non-adherence within the population.[2] In impoverished nations, including many in sub-Saharan Africa, this finding suggests a less-than-promising outcome to the HIV crisis. Truly, there remains a need for the development of novel therapeutics capable of preventing *de novo* infection or facilitating cure.

To date, only two individuals, the Berlin and London Patient, are considered either cured or in long-term remission.[3–5] In both instances, individuals received hematopoietic stem cell transplants from homozygous CCR5Δ32/32 donors, which may have prevented entry of CCR5-tropic virus into the transplanted CD4 T cells due to abrogation of co-receptor binding. Despite two undeniable successes in the field of cure research, this approach is not feasible at scale. The transplant procedure-associated risks (i.e. drug toxicity, opportunistic infections, graft rejection) greatly outweigh the necessity to take lifelong cART. Furthermore, in certain individuals receiving treatment, there are documented cases of post-treatment viral rebound or even death.[6–8] Moreover, these regimens require advanced medical training and facilities that may not be available or are limited in many regions, such as in sub-Saharan Africa. Despite the drawbacks, such studies have provided tantalizing evidence that cure is, in fact, achievable, although its usefulness is limited on a global scale.

A major hurdle to achieving HIV-1 cure is the prevalence of stably integrated provirus within host memory CD4 T cells. These reservoirs can exhibit high stability ($t_{1/2}$=44 months), indicating that latent pools are unlikely to extinguish naturally during continuous antiretroviral treatment.[9] As these cells are transcriptionally inactive, they do not produce viral RNA and proteins essential for recognition by the host immune system. Although many latent proviruses harbour large internal deletions and/or non-functional mutations, a small proportion (~2.4%) are replication competent and represent primary targets for curative strategies.[10] Many latency reversing drugs, such as HDACi, aim to overcome key epigenetic barriers, including chromatin condensation or integration into sites of low transcriptional activity. Despite *in vitro* success, many of these regimens have failed to translate into an efficacious LRA, *in vivo.*[11–13] Alternatively, PKC regimens suffer from high toxicity and low therapeutic index, *in vivo*.[14] More recent research has suggested that dendritic cell-based therapeutic regimens may be more efficacious and better tolerated.[15] This supports the finding that latency reversal within the

memory CD4 T cell reservoir is best achieved through TCR stimulation with HIV-derived peptides.[16,17] Importantly, the timing of ART initiation is crucial, as seeding of the replication competent reservoir occurs around this time.[18] Based on these findings, we hypothesized that latency reversal be optimally achieved using a virus-like particle formulation encompassing the host quasi-species immediately prior to cART initiation.

5.2 Development of a highly diverse virus-like particle formulation

Killed/attenuated vaccines have shown success in the fight against many communicable diseases. However, concerns are still present for attenuated vaccines, which have the potential to revert back towards virulence.[19] Additionally, inactivation with AT-2 or UV irradiation may alter the antigenicity and glycoprotein conformation of the vaccine formulation.[20] Therein lies a need to develop safer, more antigenic formulations. To address this, we developed a highly heterogenous, sub-type B virus-like particle formulation (Het_B_ACT-VEC) for use in therapeutic vaccine regimens. ACT-VEC is designed to act as a transcriptional 'shock' as part of a 'shock-and-kill' strategy for HIV-1 eradication.

Our ACT-VEC formulation aims to overcome safety barriers that are commonly associated with particle-based vaccine formulations, while conveying the maximum number of unique peptides for MHC-mediated TCR engagement. Firstly, ACT-VEC lacks a functional 5' LTR, including the Tat-transactivating response region. These particles were shown to be non-infectious and unable to complete reverse transcription.[20,21] Secondly, viral RNA was removed from particles through mutation of stem loop 1 (dS.1) in the RNA packaging element, thus abrogating RNA encapsidation. Despite these alterations, ACT-VEC can still translate the full HIV-1 proteome and fully assemble into viral particles. In support of this, ACT-VEC formulations had readily detectable quantities of p24 and RT production.

Importantly, ACT-VEC is developed using a yeast-based recombination system that can produce vectors representative of the entire host quasi-species. To increase heterogeneity within the formulation, ACT-VEC was created using the quasi-species formulation from five chronically infected individuals immediately prior to cART initiation. In the context of 'shock-and-kill', this provides more peptide antigens that can be recognized by HIV-specific TCRs. This is notably

different from pooled peptide 'shock' strategies, which are typically representative of a consensus subtype sequence but lack mutational variants. Moreover, VLPs maintain the viral proteins' native conformations, as opposed to pooled peptides. These differences likely contribute to increased uptake, processing, and antigenicity of VLP-based regimens.

This chapter identifies potential advantages of a heterogenous virus-like particle vaccine formulation over traditional whole-virus or peptide-based strategies. ACT-VEC was developed as a safe alternative to attenuated vaccines, lacking the functional machinery required for *in vivo* replication. Based on these findings, we now evaluate ACT-VEC's ability to induce transcriptional reactivation as part of a therapeutic strategy for HIV-1 cure.

5.3 ACT-VEC is a potent reactivator of transcriptionally quiescent provirus

Latency is maintained by a series of epigenetic factors and, in developing an LRA, these factors must be adequately addressed. During the viral integration event, HIV-1 typically inserts into regions of heterochromatin exhibiting low transcriptional activity, or within 'gene deserts' devoid of translational products. TCR-mediated reactivation shows potential in latency-reversing regimens, as downstream signalling leads to upregulation of transcription factors such as, NF-kB, NFAT, AP-1, and p-TEFb, which enhance mRNA transcription.[16,22] The Het_B_ACT-VEC formulation, described previously, aims to induce maximal reactivation of memory CD4 T cells expressing HIV-specific TCRs.

In this study, ACT-VEC- or LRA-mediated transcriptional reactivation was evaluated in CD4 T cells collected from individuals diagnosed and cART-treated during the acute stage of infection (AC-cART). Notably, this stage represents the ideal time to achieve latency reversal because of, i) less heterogeneity within the reservoir, ii) a lower occurrence of immunological escape mutants, and iii) a reduced risk of co-infection leading to the formation of alternative reservoirs. For these studies, ACT-VEC- or peptide-pulsed MDDCs were co-cultured with autologous CD4 T cells. For LRAs that act directly on the T cell, co-cultures included un-pulsed MDDCs. Notably, ACT-VEC+MDDC potently induced transcription of HIV-1 mRNA to a greater extent than observed with alternative latency reversing monotherapies, including HDACi and PKC agonists. Importantly, use of the latently infected J-Lat 6.3 cell line confirmed that all

therapeutics (HDACi, PKC agonist) were used at effective doses consistent with the literature. Moreover, these studies confirmed that ACT-VEC-based latency reversal is dependent on DC-T cell contact, as presentation of ACT-VEC to J-Lat cells alone was unable to induce transcriptional reactivation. Furthermore, to confirm that latency reversal was not a direct result of maturational differences within the MDDC population, we showed that all MDDC-pulsed conditions expressed similar levels of CD40, CD80, and CD83.

To determine the frequency of latently infected cells within our AC-cART donors, we utilized deep gene sequencing on induced, propagating virus. This data suggests that approximately 14 CD4 T cells/million are harbouring latent, potentially replication-competent, provirus. This falls within the estimated range, 1-60 cells/million, suggested by the literature.[23–25] Interestingly, a highly sensitive, fluorescent *in situ* hybridization (FISH) assay using PMA/Ionomycin detected a frequency of 3.56 latently infected CD4 T cells/million.[26] All-in-all, this may suggest that ACT-VEC potently induces reactivation to a greater degree than 'gold standard' latency reversing strategies, although FISH/Flow analysis using ACT-VEC is required to verify this.

It is important to acknowledge that, despite providing an efficacious 'shock', reduction of the latent reservoir also requires a mechanism for 'kill'. Therefore, previous studies performed by our group have determined that ACT-VEC+MDDC was sufficient to induce Granzyme B production in a co-culture containing CD4 and CD8 T cells.[20] This provides evidence that ACT-VEC-mediated reactivation could be accompanied by MHC-I recognition, thus facilitating a cytotoxic 'kill' response. Importantly, several pre-clinical studies have implicated DC-mediated viral antigen presentation in the killing of latently infected cells.[27–29] This evidence indicates that ACT-VEC may function as a 'shock' and 'kill', both of which are necessary for reservoir depletion and cure.

This chapter describes our ability to potently induce latency reversal using Het_B_ACT-VEC in AC-cART donors. ACT-VEC-mediated latency reversal resulted in readily detectable mRNA in culture supernatants, and out-performed many clinically relevant mono- and dual-therapies. Importantly, our VLP formulation displayed low antigenicity, suggesting that the cellular subset being reactivated was limited to CD4 T cells with HIV-specific TCRs. As most individuals are

diagnosed and cART-treated during the chronic stage of infection, our next study aims to evaluate latency reversal during this time.

5.4 Inter- and intra-subtype ACT-VEC induces latency reversal during chronic infection

Because most HIV-infected individuals are diagnosed and treated during the chronic stage of infection, developing vaccines that can address latency during this stage are of primary importance. Due to the prolonged period of uncontrolled infection, chronically-infected individuals can exhibit a host of complications that exacerbate the difficulties associated with reservoir reactivation. As infection continues in the absence of cART-mediated viral suppression, the latent reservoir has an opportunity to expand indefinitely. Moreover, mutational variants contribute to increased heterogeneity of the viral quasi-species. Since the latent reservoir is thought to be primarily established in HIV-specific CD4 T cells, any variations present within the viral quasi-species may also reflect novel targets for recognition and infection. Additionally, prolonged periods of inflammation may contribute to an increased occurrence of cellular anergy and/or dysfunction.[30,31] The combination of the aforementioned issues represents a barrier to curative research and, to date, have remained relatively unaddressed.

Previous studies, both from our group and others, have provided evidence that the primary reservoir resides within CD4 T cells harboring HIV-specific TCRs.[32,33] Following success in the acute stage of infection, we evaluated inter- (ACT-B) and intra-subtype (ACT-D) formulations for their ability to induce transcriptional reactivation in chronically-infected donors. Because diversity between subtypes can vary by as much as 35%, we hypothesized that optimal reactivation would occur in the context of a highly heterogenous, intra-subtype (ACT-D) formulation. Surprisingly, ACT-B- and ACT-D-pulsed MDDC resulted in similar levels of reactivation. This could prove significant, as it suggests that TCR-induced transcriptional reactivation may be mounted against immunodominant epitopes. Importantly, despite similar potency relative to other strategies (HDACi, PKC agonist), only ACT-VEC formulations could consistently induce latency reversal from all donor-derived samples. Pooled HIV peptides were also unable to compete with ACT-VEC formulations. This is likely due to several shortcoming associated with peptide-based strategies, including i) poorer uptake and presentation of

peptides, ii) poor peptide stability, and iii) a lack of genetic diversity. Overall, ACT-B/D were consistently among the most potent inducers of HIV latency while still maintaining low antigenicity.

It is noteworthy that malarial antigens and CTF (CMV/tetanus/flu) were both able to induce latency reversal within ChrD_cART donors. As is expected with chronic infection, this almost surely suggests that alternative reservoirs have been established in CD4 T cells with non-HIV-specific TCRs. In the future, deep gene sequencing of induced outgrowth virus will help us better characterize these reservoirs, in addition to HIV-specific reservoirs.

Herein, we demonstrate that inter- and intra-subtype ACT-VEC formulations can effectively induce latency reversal within ChrD-cART donors. The data further suggests that stimulation through the TCR may be preferentially mounted against immunodominant epitopes present within both subtype formulations. Despite showing that the reservoir is primarily established in HIV-specific CD4 T cells, transcriptional reactivation was also achieved using malarial and CTF peptides, suggesting that alternative reservoirs are present. This study will continue with deep gene sequencing of outgrowth virus, and comparative analysis between conditions and donor cohorts.

5.5 ACT-VEC as a therapeutic vaccine candidate

In previous chapters, we describe the development and evaluation of inter- and intra-subtype ACT-VEC in the context of donor samples isolated during both the acute and chronic stages of infection. In these studies, we conclude that a single round of ACT-VEC+MDDC stimulation can induce transcriptional reactivation of CD4 T cells exhibiting HIV-specific TCRs, as detected via culture supernatant RNA. Additionally, ACT-VEC exhibits minimal antigenicity relative to other latency reversing agents. Because of its low toxicity and potent ability to induce cellular transcription, *in vitro*, our VLP formulation merits further investigation *in vivo*. In order to recapitulate our findings *in vivo*, we must first address several important details, including i) *in situ* targeting, ii) adjuvanting, and iii) determining the route of administration.

ACT-VEC, in its current form, is a promising dendritic cell-based therapeutic vaccine candidate. Briefly, virus-like particles are phagocytosed and processed by dendritic cells before

presentation in the context of MHC-I/II, thus leading to activation of T cells with cognate antigen receptors. Interestingly, studies using heat-inactivated, autologous virus support that DC-mediated approaches are well-tolerated and contribute to a reduction in setpoint viral load.[34,35] Unfortunately, the usefulness of DC-based vaccines is limited because of the requirement to culture and pulse autologous DCs. This intrinsic feature limits global rollout of DC-based therapeutics, as the current process is expensive and patient-specific. Furthermore, different APC subsets will possess different antigen-processing capabilities, thus altering the efficacy of a vaccine candidate. To overcome these pitfalls, a DC-based therapeutic candidate requires *in situ* targeting. In the context of ACT-VEC, our group has utilized antibody-functionalized nanoparticles displaying anti-C type lectin receptor (DC-SIGN, Langerin) and/or anti-complement receptor (CD11b, CD11c)(data not shown). Interestingly, DC-SIGN, which is highly prevalent on immature DCs, has already been used for *in vivo* targeting of cancer therapeutics to DCs.[36] Moreover, VLPs that interact with C-type lectin receptors are also delivered to T cells *in trans,* thus providing alternative avenues for latency reversal in the context of an ACT-VEC+adjuvant system.[37] The ability to target our ACT-VEC particles to DCs will greatly increase the relevance of our therapeutic by overcoming key economic and logistical barriers.

Utilization of a virus-like particle formulation confers numerous advantages in respect to antigen design, presentation, and immune responsiveness. For example, VLPs express HIV-1 immunogens in their native conformation. In the case of the viral glycoprotein, gp120, the high resemblance between VLP and wildtype HIV-1 can beneficially induce a potent neutralizing antibody response. This is contrary to soluble gp120 monomeric/trimeric formulations, which have largely failed to elicit protection.[38] Even attenuated/killed whole-virus strategies fail to present glycoproteins in their native conformation, largely due to the chemical- and heat-inactivation processes.[38] To increase protection using VLPs, our team has evaluated a series of point mutations to induce greater viral glycoprotein expression and thus facilitate B cell receptor (BCR) cross-linking. Additionally, as a therapeutic, the particulate nature of a VLP can facilitate optimal uptake and processing by APCs, thus leading to stronger humoral and cell-mediated immune responses.[39,40] In ACT-VEC, the adaptive immune response is further

strengthened due to heterogeneity of the viral immunogens, which can subsequently engage with a greater number of TCRs via MHC-I/II. Interestingly, the inter-subtype functionality of ACT-VEC (refer to Chapter 4) may further suggest that identification and incorporation of immunodominant peptides would beneficially impact the vaccine's efficacy.

Up until this point, ACT-VEC has been administered as a single-dose, however, most vaccines require multiple immunizations to convey efficacy. This is the foundational reason for prime-boost regimens. Despite this, the effect of ACT-VEC as part of a homologous/heterologous prime-boost regimen is currently unknown. Traditionally, a vaccine is delivered multiple times through a homologous prime-boost regimen. Still, more recent findings indicate that administration of the same antigen(s) using different delivery modalities can be more immunogenic.[41] The rationale for a heterologous prime-boost strategy is that Env glycoproteins may induce a potent humoral response, but fail to induce cellular activation; alternatively, a recombinant vector expressing HIV-1 antigens may induce a cell-mediated immune response, but fail to produce neutralizing antibodies. Therefore, a heterologous prime-boost strategy can typically experience the benefit of both humoral and cell-mediated immunity.[41,42] In the context of ACT-VEC, our research has identified potent cell-mediated immune responsiveness, however, the antibody-producing capacity of our VLP formulation remains unknown. Interestingly, increasing the amount of Env glycoprotein on the surface of ACT-VEC might be sufficient to induce BCR cross-linking and antibody production. As such, ACT-VEC may perform well as part of a homologous prime-boost strategy, as well as a heterologous regimen.

Despite the promise of our initial ACT-VEC formulation, we remain interested in strategies to improve upon the vector's antigenicity. Previously, we compared the antigenic differences between viral particles (VPs) and virus-like particles, including ACT-VEC. Unsurprisingly, the presence of viral RNA in our VP formulation contributed to an increase in antigenicity relative to VLPs. With this knowledge, our team developed a packaging-competent immunostimulatory DNA vector that can be transcribed *in vivo* through utilization of a CMV promoter. These adjuvants contain 'GC' and 'AU' rich motifs that bias the immune response towards IFN-α and TNF-α production, respectively. Based on preliminary studies looking at NF-kB- and interferon regulatory factor (IRF) expression, we found that VLP+adjuvant were more antigenic than either

VP and VLP, alone. Presumably, the presence of packaged RNA enhances recognition through innate immune-sensing molecules, such as toll-like receptor 7. Overall, incorporation of immune-stimulatory adjuvants may enhance the cell-mediated immune response and further improve ACT-VEC.

5.6 Concluding Remarks

The preceding work outlines the development and evaluation of ACT-VEC formulations derived from chronically-infected subtype B and D donors. In the future, work will initially focus on expanding the chronic study to incorporate more donors. Currently, we have samples available for nine ChrD-cART donors, with access to additional samples, if necessary. This work will be performed in collaboration with colleagues at Johns Hopkins University, where they will be performing quantitative viral outgrowth assays (qVOA). Additionally, outgrowth virus from ChrD-cART donors will be sent for Illumina deep gene sequencing and subsequently compared to autologous proviral samples. A collaboration with Dr Daniel Kaufmann will allow us to evaluate ACT-VEC in the context of their fluorescent *in situ* hybridization (FISH) flow cytometric assay. The FISH/Flow assay will provide us with single-cell analysis and more accurate information regarding the frequency of latently infected cells within our donor cohorts.

Up until now, most investigations have focused on ACT-VEC in the context of a highly specific LRA. However, several studies have indicated that DC-based strategies are capable of inducing 'kill' via immune cytotoxic mechanisms.[15,27] Interestingly, a preliminary study has shown that ACT-VEC induces Granzyme B production in an MDDC+CD4+CD8 co-culture assay.[20] To expand on this finding, a future investigation will aim to complete the Granzyme B 'killing' assay. Ultimately, the aim of these studies will be to associate 'kill' with an overall reduction in reservoir size.

Upon summation of the *in vitro* studies, we will continue to evaluate ACT-VEC *in vivo*. To do this, we will utilize a non-human primate model of infection to test the *in vivo* efficacy of heterologous, SIV-derived ACT-VEC (ACT-VEC$_{SIV}$). Pre-clinical analysis will determine the efficacy of ACT-VEC$_{SIV}$ to i) induce transcriptional reactivation of SIV-specific T cells, and ii) reduce the overall size of the reservoir. At this stage, toxicity and off-target effects, if any, will also be

noted. At the current time, it is unknown whether ACT-VEC$_{SIV}$ will be administered as part of a homologous or heterologous prime-boost protocol, nor has the route of administration been confirmed. Assuming that these initial *in vivo* studies are well-tolerated and at least modestly efficacious, we will continue testing ACT-VEC in the context of human clinical trials.

Herein, we describe ACT-VEC as a highly specific, albeit potent inducer of transcriptional reactivation. In both cohorts of infection, ACT-VEC induces latency reversal to a similar or greater extent than clinically relevant LRAs, including HDACi, PKC agonists, and TLR agonists. ACT-VEC exhibits low antigenicity, suggesting that it will be well tolerated *in vivo*. Moreover, successful reactivation using inter- and intra-subtype ACT-VEC formulations may suggests that TCR-mediated reactivation is preferentially mounted against immunodominant epitopes present within both subtypes. Overall, ACT-VEC represents a novel strategy for latency reversal that merits further investigation in the context of therapeutic vaccination.

5.7 References

1. UNAIDS. Fact Sheet - Global AIDS Update 2019. *UNAIDS* (2019).

2. Burch, L. S. *et al.* Socioeconomic status and treatment outcomes for individuals with HIV on antiretroviral treatment in the UK: cross-sectional and longitudinal analyses. *Lancet Public Heal.* (2016). doi:10.1016/S2468-2667(16)30002-0

3. Allers, K. *et al.* Evidence for the cure of HIV infection by CCR5Delta32/Delta32 stem cell transplantation. *Blood* **117**, 2791–2799 (2011).

4. Hutter, G. *et al.* Long-term control of HIV by CCR5 delta32/delta32 stem-cell transplantation. *N. Engl. J. Med.* (2009). doi:10.1056/NEJMoa0802905

5. Gupta, R. K. *et al.* HIV-1 remission following CCR5Δ32/Δ32 haematopoietic stem-cell transplantation. *Nature* (2019). doi:10.1038/s41586-019-1027-4

6. Hütter, G. More on shift of HIV tropism in stem-cell transplantation with CCR5 delta32/Delta32 mutation. *New England Journal of Medicine* (2014). doi:10.1056/NEJMc1412279

7. Kordelas Lambros，Jens Verheyen，Stefan Esser. Shift of HIV Tropism in Stem-Cell Transplantation with CCR5 Delta32 Mutation. *N. Engl. J. Med.* (2014). doi:10.1056/nejmc1407921

8. Tebas, P. *et al.* Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N. Engl. J. Med.* (2014). doi:10.1056/NEJMoa1300662

9. Siciliano, J. D. *et al.* Long-term follow-up studies confirm the stability of the latent

reservoir for HIV-1 in resting CD4+ T cells. *Nat. Med.* **9**, 727–728 (2003).

10. Bruner, K. M. *et al.* Defective proviruses rapidly accumulate during acute HIV-1 infection. *Nat. Med.* (2016). doi:10.1038/nm.4156

11. Sagot-Lerolle, N. *et al.* Prolonged valproic acid treatment does not reduce the size of latent HIV reservoir. *AIDS* (2008). doi:10.1097/QAD.0b013e3282fd6ddc

12. Wei, D. G. *et al.* Histone Deacetylase Inhibitor Romidepsin Induces HIV Expression in CD4 T Cells from Patients on Suppressive Antiretroviral Therapy at Concentrations Achieved by Clinical Dosing. *PLoS Pathog.* **10**, (2014).

13. Lucera, M. B. *et al.* The Histone Deacetylase Inhibitor Vorinostat (SAHA) Increases the Susceptibility of Uninfected CD4+ T Cells to HIV by Increasing the Kinetics and Efficiency of Postentry Viral Events. *J. Virol.* **88**, 10803–10812 (2014).

14. Gutiérrez, C. *et al.* Bryostatin-1 for latent virus reactivation in HIV-infected patients on antiretroviral therapy. *AIDS* (2016). doi:10.1097/QAD.0000000000001064

15. Kristoff, J., Rinaldo, C. R. & Mailliard, R. B. Role of dendritic cells in exposing latent HIV-1 for the kill. *Viruses* (2019). doi:10.3390/v12010037

16. Mohammadi, P. *et al.* Dynamics of HIV Latency and Reactivation in a Primary CD4+ T Cell Model. *PLoS Pathog.* **10**, (2014).

17. Shete, A. *et al.* Short Communication: HIV Antigen-Specific Reactivation of HIV Infection from Cellular Reservoirs: Implications in the Settings of Therapeutic Vaccinations. *AIDS Res. Hum. Retroviruses* **28**, 835–843 (2012).

18. Abrahams, M. R. *et al.* The replication-competent HIV-1 latent reservoir is primarily established near the time of therapy initiation. *Sci. Transl. Med.* (2019). doi:10.1126/scitranslmed.aaw5589

19. Chakrabarti, L. A. *et al.* A Truncated Form of Nef Selected during Pathogenic Reversion of Simian Immunodeficiency Virus SIVmac239 nef Increases Viral Replication. *J. Virol.* (2003). doi:10.1128/jvi.77.2.1245-1256.2003

20. Pankrac, J. *et al.* A heterogeneous human immunodeficiency virus-like particle (VLP) formulation produced by a novel vector system. *npj Vaccines* **3**, 2 (2018).

21. Dudley, D. M. *et al.* A novel yeast-based recombination method to clone and propagate diverse HIV-1 isolates. *Biotechniques* (2009). doi:10.2144/000113119

22. Shan, L. *et al.* Stimulation of HIV-1-Specific Cytolytic T Lymphocytes Facilitates Elimination of Latent Viral Reservoir after Virus Reactivation. *Immunity* (2012). doi:10.1016/j.immuni.2012.01.014

23. Eriksson, S. *et al.* Comparative Analysis of Measures of Viral Reservoirs in HIV-1 Eradication Studies. *PLoS Pathog.* (2013). doi:10.1371/journal.ppat.1003174

24. Ho, Y. C. *et al.* XReplication-competent noninduced proviruses in the latent reservoir

increase barrier to HIV-1 cure. *Cell* (2013). doi:10.1016/j.cell.2013.09.020

25.   Procopio, F. A. *et al.* A Novel Assay to Measure the Magnitude of the Inducible Viral Reservoir in HIV-infected Individuals. *EBioMedicine* (2015). doi:10.1016/j.ebiom.2015.06.019

26.   Baxter, A. E. *et al.* Single-Cell Characterization of Viral Translation-Competent Reservoirs in HIV-Infected Individuals. *Cell Host Microbe* (2016). doi:10.1016/j.chom.2016.07.015

27.   Macatangay, B. J. C. *et al.* Therapeutic Vaccination with Dendritic Cells Loaded with Autologous HIV Type 1-Infected Apoptotic Cells. *J. Infect. Dis.* (2016). doi:10.1093/infdis/jiv582

28.   Jacobson, J. M. *et al.* Dendritic cell immunotherapy for HIV-1 infection using autologous HIV-1 RNA: A randomized, double-blind, placebo-controlled clinical trial. *J. Acquir. Immune Defic. Syndr.* (2016). doi:10.1097/QAI.0000000000000926

29.   Lu, W., Wu, X., Lu, Y., Guo, W. & Andrieu, J. M. Therapeutic dendritic-cell vaccine for simian aids. *Nat. Med.* (2003). doi:10.1038/nm806

30.   Meyaard, L., Schuitemaker, H. & Miedema, F. T-cell dysfunction in HIV infection: anergy due to defective antigen-presenting cell function? *Immunol. Today* (1993). doi:10.1016/0167-5699(93)90279-T

31.   Sousa, A. E., Carneiro, J., Meier-Schellersheim, M., Grossman, Z. & Victorino, R. M. M. CD4 T Cell Depletion Is Linked Directly to Immune Activation in the Pathogenesis of HIV-1 and HIV-2 but Only Indirectly to the Viral Load. *J. Immunol.* (2002). doi:10.4049/jimmunol.169.6.3400

32.   Douek, D. C. *et al.* HIV preferentially infects HIV-specific CD4+ T cells. *Nature* **417**, 95–98 (2002).

33.   Demoustier, A. *et al.* In patients on prolonged HAART, a significant pool of HIV infected CD4 T cells are HIV-specific. *AIDS* **16**, 1749–1754 (2002).

34.   García, F. *et al.* A dendritic cell-based vaccine elicits T cell responses associated with control of HIV-1 replication. *Sci. Transl. Med.* (2013). doi:10.1126/scitranslmed.3004682

35.   García, F. *et al.* A therapeutic dendritic cell-based vaccine for HIV-1 infection. *J. Infect. Dis.* (2011). doi:10.1093/infdis/jiq077

36.   Aarnoudse, C. A., Vallejo, J. J. G., Saeland, E. & Van Kooyk, Y. Recognition of tumor glycans by antigen-presenting cells. *Current Opinion in Immunology* (2006). doi:10.1016/j.coi.2005.11.001

37.   Geijtenbeek, T. B. H. *et al.* DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* (2000). doi:10.1016/S0092-8674(00)80694-7

38.   Gao, Y., Wijewardhana, C. & Mann, J. F. S. Virus-like particle, liposome, and polymeric particle-based vaccines against HIV-1. *Frontiers in Immunology* (2018).

doi:10.3389/fimmu.2018.00345

39.   Gamvrellis, A. *et al.* Vaccines that facilitate antigen entry into dendritic cells. *Immunology and Cell Biology* (2004). doi:10.1111/j.0818-9641.2004.01271.x

40.   Slütter, B. & Jiskoot, W. Sizing the optimal dimensions of a vaccine delivery system: A particulate matter. *Expert Opinion on Drug Delivery* (2016). doi:10.1517/17425247.2016.1121989

41.   Lu, S. Heterologous prime-boost vaccination. *Current Opinion in Immunology* (2009). doi:10.1016/j.coi.2009.05.016

42.   Lu, S. Combination DNA plus protein HIV vaccines. *Springer Seminars in Immunopathology* (2006). doi:10.1007/s00281-006-0028-1

Chapter 6

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SPRINGER NATURE

A heterogeneous human immunodeficiency virus-like particle (VLP) formulation produced by a novel vector system

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Chapter 7 - Curriculum Vitae

Joshua Pankrac

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Education

PhD in Microbiology and Immunology	**Pending Completion**
Schulich School of Medicine and Dentistry, London, ON	

Honours Bachelor of Science	**April 2016**
Western University, London, ON	
Double Major in Medical Science and Biology (Hons.)	

Research Experience

Graduate Research Assistant (Lab of Dr. E.J. Arts and Dr. J. Mann)	**2017-Present**
Schulich School of Medicine, London, Ontario	

Research Technician	**2016**
Schulich School of Medicine, London, Ontario	

Undergraduate Research Assistant	**2014-2016**
Schulich School of Medicine, London, Ontario	

Teaching Experience

Graduate Teaching Assistant (GTA)	**2018**
Microbiology and Immunology 2500B	

Graduate Teaching Assistant (GTA)	**2019**
Microbiology and Immunology 3620G	

Laboratory-Based Training

• Rahul Pawa (M.Sc. Candidate)	**2016-Present**
• Renata Ceccacci (4th Year Undergraduate)	**2019-Present**
• Chanuka Wijewardhana (M.Sc.)	**2016-2019**
• Seth Kibel (4th Year Undergraduate)	**2018-2019**

Publications

Joshua Pankrac, Katja Klein, Jamie F.S. Mann (2017). *Eradication of HIV-1 latent reservoirs through therapeutic vaccination.* AIDS Research and Therapy; **14**:45.

Joshua Pankrac, Katja Klein, Paul F. McKay, Deborah F. L. King, Katie Bain, Jason Knapp, Tsigereda Biru, Chanuka N. Wijewardhana, Rahul Pawa, David H. Canaday, Yong Gao, Sarah Fidler, Robin J. Shattock, Eric J. Arts & Jamie F. S. Mann (2018). *A heterogeneous human immunodeficiency virus-like particle (VLP) formulation produced by a novel vector system.* npj Vaccines; **3**:2. doi:10.1038/s41541-017-0040-6.

Jamie F.S. Mann, **Joshua Pankrac**, Katja Klein, Paul F. McKay, Deborah F.L. King, Richard Gibson, Chanuka N. Wijewardhana, Rahul Pawa, Jodi Meyerowitz, Yong Gao, David H. Canaday, Mariano Avino, Art F.Y. Poon, Caroline Foster, Sarah Fidler, Robin J. Shattock and Eric J. Arts. *A targeted reactivation of latent HIV-1 using an activator vector.* **Manuscript Accepted.**

Joshua Pankrac, Jamie F.S. Mann, Emmanuel Ndashimye, Rahul Pawa, Renata Ceccacci, Eric Arts. *Potent reactivation of latent provirus using a heterogenous virus-like particle formulation.* **Manuscript in preparation.**

Joshua Pankrac, Chanuka Wijewardhana, Rahul Pawa, Katja Klein, Eric Arts, Jamie F.S. Mann. *Virally packaged RNA in virus-like particle vaccines enhance antigenicity and augment latency reversal of HIV-1.* **Manuscript in preparation.**

Conferences and Presentations

Infection and Immunity Research Forum (IIRF)	**2019**
Oral Presentation	
Canadian Association for HIV Research (CAHR)	**2018**
Poster Presentation	
Canadian Association for HIV Research (CAHR)	**2017**
Poster Presentation	
Infection and Immunity Research Forum (IIRF)	**2017**
Poster Presentation	
Infection and Immunity Research Forum (IIRF)	**2016**
Poster Presentation	
Western HIV Investigator Group (WHIG)	**2015-2019**
Oral Presentation(s)	
Canadian Association for HIV Research (CAHR)	**2015**
Poster Presentation	
American Society for Virology (ASV)	**2015**

Awards

- **Ontario Graduate Scholarship ($15 000)** — **2019-2020**
  Schulich School of Medicine and Dentistry, Western University
- **FW Luney Travel Award ($1 000)** — **2018**
  Schulich School of Medicine and Dentistry, Western University
- **Dean's List** — **2016**
  Western University
- **Leadership Education Letter of Accomplishment** — **2014**
  Leadership Education Program, Western University
- **Western Scholarship of Excellence ($2 000)** — **2012**
  Western University
- **OPG Bursary for Outstanding Achievement in Science ($500)** — **2012**
  Ontario Power Generation, Canada
- **Honourable Delegate Award (Top 1%)** — **2011**
  International Model United Nations, United Nations

Certifications

- Radiation Safety Training, *University of Western Ontario* **2019-2022**
- Comprehensive WHMIS Training, *University of Western Ontario* **2019-2022**
- Biosafety Training, *University of Western Ontario* **2018**
- Hazardous Waste Management, *University of Western Ontario* **2018**

Community Involvement

- Scientific Educator, *Regional HIV/AIDS Connection* **2020**
- Leadership Education Program (LEP) Facilitator, *University of Western Ontario* **2015-2016**
- Physiotherapy Volunteer, *St Joseph's Health Care London* **2013-2015**
- Conference Volunteer, *American Society for Virology* **2015**