# Dynamics of the HIV-1 Latent Reservoir

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**Abstract**

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Human immunodeficiency virus type 1 (HIV) has caused more than 35 million deaths world-wide and contributes significantly to the global burden of disease. Currently, the only effective treatment to suppress viral replication and prevent HIV transmission is combination antiretroviral therapy (ART), which prevents HIV from infecting new cells. Despite the efficacy of ART, long lived latently infected cells persist, with an estimated half-life of 44 months, and circulate throughout the infected host necessitating life-long treatment. These cells, known as the latent reservoir, contain an integrated form of the HIV genome (HIV DNA) that is transcriptionally silent, but can reactivate to produce virus. Therefore, interruption of ART inevitably leads to viral recrudescence stemming from the latent reservoir. Studying the latent reservoir is difficult because these cells contain no known biomarkers and do not always produce replication competent virus upon cellular activation. Additionally, latent reservoir cells are rare, and many proviral genomes contain defects that prevent them from producing replication competent virus. They do however confound efforts to measure the replication competent reservoir. Understanding the dynamics and correlates of reservoir seeding will be essential to develop novel cure strategies that target this latent

reservoir. There is limited data on the dynamics of reservoir seeding throughout HIV infection, the impact of treatment interruption on reservoir size, and whether antibodies can play a role in limiting reservoir seeding. I focused my thesis on characterizing the seeding dynamics of latent reservoir cells containing HIV DNA (HIV DNA Reservoir) to better understand when the latent reservoir was generated and how it changed following treatment interruption.

In the first part of this thesis I adapt, optimize, and validate a molecular based assay to quantitate HIV DNA from latently infected cells, as well as develop a novel cell line to detect replication competent HIV reactivated from latent reservoirs. In the second part of this thesis I demonstrate that the HIV DNA reservoir is limited by early ART and does not significantly increase following randomization to short treatment interruption in a cohort of Kenyan infants, suggesting that short treatment interruption studies may pose little risk to reservoir reseeding. I also examine the role of ADCC activity in preventing re-seeding of the latent reservoir and demonstrate that ADCC activity does not correlate with change in HIV DNA reservoir size following treatment interruption. Finally, I demonstrate that the HIV DNA reservoir is comprised mostly of viral variants circulating just prior to ART initiation, suggesting that during untreated infection the HIV DNA reservoir decays at a much faster rate than during suppressive ART. Together, these data demonstrate that the HIV DNA reservoir is limited by early ART, is not significantly reseeded with short treatment interruption, and that contrary to previous assumptions about reservoir dynamics, is decaying at a significantly faster rate pre-ART than after ART initiation, and suggest that targeting the HIV latent reservoir prior to early ART initiation may be an effective strategy to limit reservoir size, and that short treatment interruption can limit re-seeding of the latent reservoir.

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# Chapter 1. Introduction

## 1.1 HIV global epidemiology

In the early 1980's the incidence of mortality caused by opportunistic infections in young homosexual men greatly increased, leading to the discovery of a virus [1], human immunodeficiency virus type-1 (HIV), that attacked the immune system. Since the discovery of HIV, identified to be the causative agent of acquired immunodeficiency syndrome (AIDS), more than 35 million people have died from HIV across the globe. As of 2017, an estimated 36.7 million people were living with HIV [2], with the African Region having the highest incidence of HIV infection (Figure 1.1).

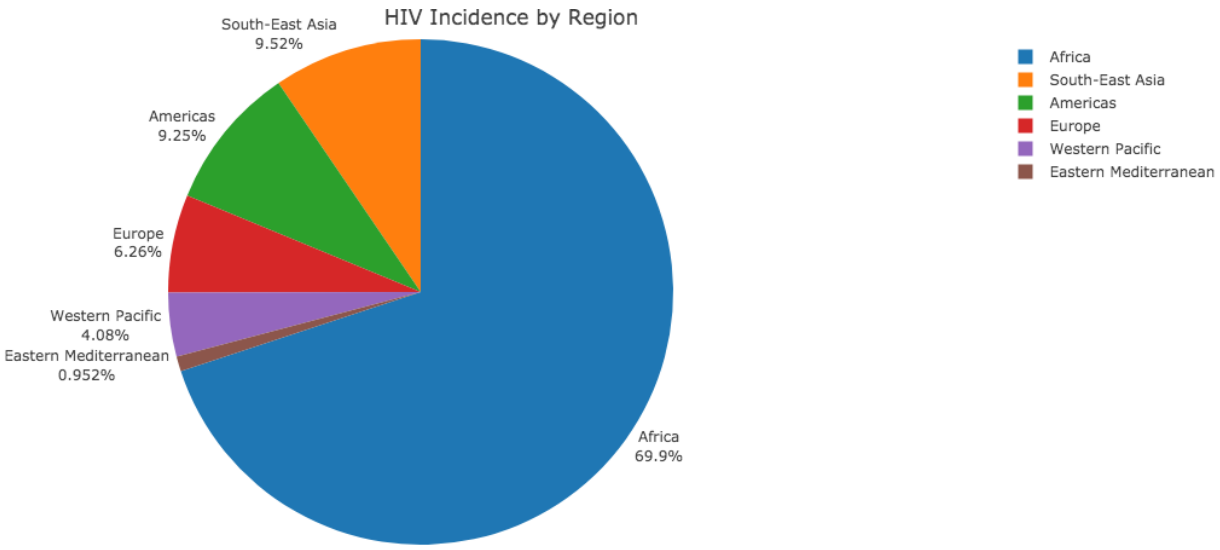


**Figure 1.1. Global HIV incidence**.
Data from UNAIDS 2017 report. Pie chart showing percentages of the 36.7 million people living with HIV in UNAIDS defined regions.

The arrival of triple drug combination antiretroviral therapy (ART) in the late 1990's greatly reduced morbidity and mortality [3, 4] and has been shown to be efficacious in preventing

infections in serodiscordant couples [5]. While ART has undoubtedly saved many lives, HIV incidence is still high with an estimated 1.8 million new infections occurring in 2017, with 180,000 infections occurring in children less than 15 years old [2]. Global efforts have focused on increasing HIV treatment services to key populations to prevent new infections, but social barriers and resource constraints have limited the efficacy of these efforts [2]. Despite these limitations these approaches are showing benefit as HIV incidence is declining, with the largest reduction in incidence in Sub-Saharan Africa.

Although HIV incidence is declining, there are still 21.7 million people accessing ART [6], and efforts to develop an HIV vaccine or cure have been largely unsuccessful. While ART is sufficient to reduce viral load and prevent new infections, treatment duration is lifelong, and ART treated individuals have been shown to have a higher risk of mortality and age related morbidity [7]. It is paramount that an HIV cure, leading to complete elimination of the virus in a person, be developed to reduce the burden of disease and prevent further HIV infection. This is not something ART alone can do.

## 1.2 HIV-1 lifecycle

HIV is an enveloped, positive sense, single stranded RNA virus in the *Retroviridae* family. It has zoonotic origins derived from a cross-species transmissions between chimpanzees and humans [8] and encodes 19 proteins from 9 genes. HIV binds to a cell when the envelope protein on the surface of the virion binds with the host receptor CD4 [9, 10], which is present on CD4+ T cells, monocytes, macrophages, and dendritic cells. HIV envelope interacts with the host co-receptor CXCR4 or CCR5[11] which is present on CD4+ T cells, monocytes, macrophages, and

dendritic cells. After binding to CD4, HIV envelope interacts with the host co-receptor CXCR4 or CCR5 mediating a cascade of conformational changes leading to fusion of the virus with the host cell [12] and egress of the viral genome into the host cell cytoplasm. The viral derived enzyme reverse transcriptase, which is error prone and responsible for HIV's high rate of mutation, transcribes the RNA into double stranded DNA [13]. The viral genome is then imported into the host nucleus where the viral protein Integrase mediates integration of the provirus preferentially into host sites of active transcription [14]. This process results in lifelong infection of the cell. Once integrated, host transcription and translation machinery can drive the production of HIV virions capable of spreading infection to new cells.

## 1.3 HIV sequence diversity

HIV Type 1 group M consists of 9 major subtypes which share 70-90% sequence similarity [15]. Globally in 2015, subtype C was the predominant subtype, accounting for >40% of the HIV burden, but in many countries multiple subtypes co-circulate and contribute to the observed rise in diversity due to recombinant subtype HIV infection [16]. HIV diversity is driven by both viral and host factors.

The viral protein reverse transcriptase, which synthesizes double-stranded proviral DNA using viral RNAs as templates, is low-fidelity [17] and does not contain a subunit for proofreading which causes an estimated error rate of $2x10^{-5}$ per nucleotide per replication cycle [13]. Furthermore, strand transfer between RNA templates during reverse transcription reverse can lead to recombination between HIV templates, [18]. This can drive HIV genetic shift when a cell is infected with two genetically distinct viruses and a virus with each genome is packaged in a single virus. In the subsequent infection, recombination can occur, creating a provirus with elements

from each genome. Along with HIV's high rate of mutation, the high rate of virus turnover ($10^{10}$ viral particles per day) during infection facilitates the diversification of the HIV quasispecies *in vivo* [15]. On the host side, APOBEC family proteins packaged with viral genomes, catalyze cytosine to uracil editing, and cause mutation of the viral genome [19]. While many APOBEC mediated mutations can result in virus variants that are replication-deficient, some can be sublethal and contribute to HIV diversity [20]. Overall, during infection the HIV genome exhibits a divergence of 0.3-1% per year from the genome of the infecting strain, depending on the genome region [21, 22].

## 1.4 Antiretroviral therapy and HIV RNA and DNA dynamics

Combination antiretroviral therapy (ART) targets different steps in the viral life cycle pre- and post- HIV integration. The four classes of drugs that are commonly being administered to treat HIV are: nucleoside analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside analogue reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and fusion inhibitors [23]. Administration of ART results in a biphasic decay of HIV RNA with the first phase of decay attributed to the clearance of productively infected CD4+ T cells and the second phase attributed to longer lived cells such as tissue resident macrophages [24]. While HIV RNA levels decay to levels that are undetectable by clinical assays, HIV DNA levels have been shown to be remarkably stable after the initial decay with an estimated half-life of ~12 years [25]. Furthermore, it has been demonstrated that the majority of HIV DNA present during suppressive ART is replication deficient due to large deletions caused by a "copy choice" mechanism of RNA strand transfer during reverse transcription [26] or other mutations in the genome caused by methods discussed in 1.3. Thus, a rare subset of HIV DNA is replication competent, with an

estimated half-life of 44 months, and preserved in the latent reservoir during suppressive ART [27].

## 1.5 The HIV latent reservoir

The main target cells of HIV infection are activated CD4+ T cells. Resting CD4+ T cells are refractory to HIV infection due to the low concentration of dNTPs caused by the restriction factor SAMHD1 which limits the available pool of nucleotides necessary for HIV reverse transcription [28]. HIV has been shown to infect other cell types that express the receptor CD4 and the co-receptor CCR5 or CXCR4, such as monocytes, macrophages, and dendritic cells [29] [30]. While HIV can infect these cell types, the majority of these cells do not exhibit the longevity of CD4+ T cells and are not thought to be major contributors to the HIV latent reservoir [31].

When activated CD4+ T cells are infected with HIV, they are eliminated quickly due to viral cytopathic effects [32] or bystander cell pyroptosis [33]. However, approximately one in a million infected activated CD4+ T cells survive long enough to revert from an activated state to a resting state [34]. In this resting state, the HIV provirus remains in the host genome and is transcriptionally silent, producing no viral protein, causing it to remain invisible to the host immune system [35]. These HIV infected resting CD4+ T cells comprise the major population of the HIV latent reservoir which can persist for decades despite effective ART [27]. Clonal expansion of CD4+ T cells that contain integrated provirus but are not eliminated by the immune system or viral cytopathic effects during suppressive ART contribute to this persistence [36-38]. Typically, within weeks of ART discontinuation, HIV rapidly rebounds from the latent reservoir as reactivation of virus occurs in a subset of these cells [39] necessitating lifelong ART.

## 1.6 HIV latency mechanisms

Latency can be maintained through both cellular and viral mechanisms. Cellular mechanisms of proviral latency include, but are not limited to, transcriptional interference, heterochromatin silencing, epigenetic modification, and restriction of essential transcription factors [40]. Transcriptional interference occurs when the HIV provirus integrates into an actively transcribed gene. HIV transcription is silenced due to read-through of RNA polymerase II during transcription of genes upstream of the integrated provirus [41]. Transcription of cellular genes upstream of HIV continues through the 5' LTR of the integrated HIV genome, and displaces transcription factors that mediate HIV transcription [42]. The displacement of these transcription factors causes reduced expression of HIV transcripts, promoting silencing while simultaneously increasing expression of transcripts driven by the 3' LTR [40]. Heterochromatin and epigenetic silencing occurs when nucleosomes are recruited to the LTR of the integrated viral genome. The nucleosomes prevent RNA polymerase II from associating with the LTR and initiating transcription, leading to reduced viral transcription [40]. This form of latency can be reversed through the addition of cellular activators, which promote translocation of transcription factors that bind the LTR from the cytoplasm to the nucleus, as well as exogenous Tat, which promotes elongation of viral transcripts [43]. Transcription factors that are essential to the transcription of the HIV provirus include NFκB, SP1, and NFAT [40]. These transcription factors bind the LTR of HIV and enhance transcription of the viral genes Tat and Rev, which promote transcript elongation and export from the nucleus, and thus drive the virus out of latency.

## 1.7 Measuring the HIV latent reservoir

In order to measure the HIV latent reservoir, samples must be from individuals on suppressive ART for at least 6 months to prevent quantitation of HIV in activated cell types that have yet to decay. There are currently a multitude of assays that detect different aspects of the latent reservoir; however, not one assay is able to accurately detect latent provirus capable of producing virus when activated [44-46]. The current gold standard assay is the quantitative viral outgrowth assay (QVOA), which quantitates the amount of provirus that can be induced to produce replication competent virus through cellular activation pathways in cell culture [47]. CD4+ T cells are placed in a limiting dilution series and latent provirus is induced to produce replication competent virus. The virus produced from these cells is then propagated and expanded in 3 separate additions of donor PBMCs over 21 days and latent reservoir size is quantitated by an assay that detects a HIV core protein (p24 ELISA) of the limiting dilution series. IL-2 and phytohemagglutinin (PHA) are used to stimulate CD4+ T cells into dividing; however, cellular activation is not always sufficient to induce virus production from latent provirus. Non-induced proviruses have been shown to produce replication competent virus following a second round of cellular activation [48]. Sequencing of non-induced wells revealed replication competent provirus, suggesting that the QVOA underestimates the latent reservoir by as much as 60-fold [48]. Even though the QVOA underestimates the size of the latent reservoir and is labor intensive, it provides a minimal estimate of replication competent provirus.

Molecular based assays that quantitate the size of the HIV reservoir measure total HIV DNA that is integrated into the host genome, HIV DNA that is generated during abortive

infection that is not integrated, or HIV RNA that is produced upon viral reactivation [45]. These assays typically overestimate the size of the latent reservoir as they quantitate both replication competent and replication deficient forms of the viral nucleic acids [49]. Replication deficient forms of HIV DNA include viral genomes hypermutated by APOBEC3G, as well as genomes containing large internal deletions and premature stop codons[48]. A combination of molecular based assays can better define the latent reservoir than each assay alone by quantitating and excluding replication deficient and non-integrated forms of HIV DNA such as 2-LTR Circles, which are formed when HIV DNA circularizes into an episomal form during abortive infection [44]. Even though these assays overestimate the size of the latent reservoir, they are relatively inexpensive, require little sample volume, and don't rely on cell viability; making them more attractive to studies that have limited sample availability.

More recently an assay was developed to overcome some of the limitations of the QVOA and molecular based assays. The Tat/Rev induced limiting dilution assay (TILDA) stimulates latently infected cells using similar methods to the QVOA assay, and then quantitates the latent reservoir by measuring multiply spliced RNA (msRNA) generated from the cells that are distributed in a limiting dilution format by either quantitative PCR (qPCR) or droplet digital PCR (ddPCR) [50]. This assay reduces the amount of blood needed for each assay from 120-180 mLs in the QVOA assay to ~10 mLs needed for TILDA. This assay also reduces the amount of replication deficient nucleic acid that is detected compared to molecular based assays for total HIV DNA. TILDA, while being less labor intensive and requiring less blood than the QVOA, still has the propensity to detect replication deficient forms of HIV because the presence of viral RNA does not mean the viral genome is infectious.

It also relies upon activation of the provirus by T cell stimulation and as noted above, not all cells that are capable of producing virus do so when cells are activated.

Recently, a new strategy of amplifying full length HIV from latently infected cells by single genome amplification has been employed to circumvent the re-activation step necessary for the QVOA and TILDA assays to more accurately quantify the latent reservoir [48, 51, 52]. Using limiting dilution and a near full length PCR amplification, proviral genomes are amplified and sequenced from resting CD4+ T cells. This strategy allows for quantitation that is not biased by reactivation kinetics, as well as direct sequencing of intact proviral variants. Quantitation by this method has estimated that the latent reservoir is 10-100 times greater than that estimated by QVOA, suggesting that assay optimization and development are still greatly needed to identify an assay that can accurately quantify the replication competent latent reservoir.

## 1.8 Latent reservoir seeding

The latent reservoir is seeded prior to detection of viremia in the blood. In a macaque model of infection, the latent reservoir was established within three days of exposure to SIV in the lymph node and gut mucosa, and by day seven the reservoir was established in the PBMCs [53]. This study demonstrated that the latent reservoir is established extremely early, but may be limited in size and location by early treatment. Studies in humans have found similar results. In adults, treatment during acute infection reduced the latent reservoir size compared to those treated later and in one study, early treatment led an increased incidence of post-treatment controllers who no longer required ART to suppress viremia [54]. The latent reservoir size of perinatally infected infants also appears to be

limited by early treatment, although there are few studies with limited sample size. In a randomized trial of 247 infants who were diagnosed with HIV prior to 12 weeks of age, those who were treated immediately had a lower latent reservoir size (median: 270 copies of HIV DNA/$10^6$ PBMCs) than those who deferred treatment until clinical symptoms (median: 1000 copies of HIV DNA/$10^6$ PBMCs), suggesting that treatment prior to 12 weeks of age limited the size of the latent reservoir [55]. Additional smaller studies in infants have also indicated that early treatment limits the latent reservoir size [56, 57].

There have been few studies examining when the reservoir is seeded throughout infection. Recently, deep sequencing has made it possible to identify the sequences of proviral HIV DNA; however, studies have been limited to individuals infected by virus from a single source partner, thus relying on phylogenetic differences from viral evolution during untreated infection to define timing of reservoir seeding. One study has attempted to improve temporal resolution by sequencing proviral DNA from patients with resistance mutations generated by treatment with suboptimal therapy [58]. They observed that time on suboptimal therapy and proportion of provirus harboring resistance mutations were positively correlated, suggesting that the reservoir was continuously seeded over time.

Studies of what grows out of the latent reservoir when therapy is discontinued have observed that rebound virus is the most recent common ancestor of pre-therapy virus, suggesting that the reservoir is seeded predominantly early [59]. In contrast, a study matched HIV RNA p17gag sequences from plasma during infection to HIV DNA p17gag sequences obtained from PBMCs after ART and observed that the HIV DNA most closely resembled the circulating viral associated RNA just prior to ART, suggesting that the reservoir continuously turns over and is seeded continuously [60]. Further supporting this

observation, a recent study matched outgrowth virus sequences from resting CD4+ T cells following ART initiation and to sequences from longitudinal HIV RNA; identifying that >70% of outgrowth viruses were phylogenetically closest to HIV RNA sequences identified near ART initiation [61]. Seeding dynamics of the latent reservoir and what promotes seeding is still unclear and further studies are needed to resolve whether the latent reservoir is seeded predominantly early or continuously throughout infection.

## 1.9 HIV cure studies and treatment interruption

It is thought that prevention of viral recrudescence from latent reservoirs will lead to an HIV cure. The goal of a "sterilizing" cure is to prevent viral rebound by either eliminating integrated replication competent forms of HIV, or irreversibly altering integrated HIV provirus so that it is replication deficient; while the goal of a "functional" HIV cure is to achieve HIV remission in the absence of treatment [62].

The first evidence that an HIV cure was attainable was from the case of the "Berlin Patient", an HIV infected individual who was treated for acute myeloid leukemia [63] by an allogeneic stem cell transplant from a donor who was homozygous for *CCR5* containing a 32 base pair deletion ($CCR5^{\Delta 32/\Delta 32}$), which has been shown to resist HIV infection [64]. Two HIV infected lymphoma patients from Boston treated by allogeneic stem cell transplantation from individuals homozygous for wildtype *CCR5*, which are susceptible to HIV infection, had viral rebound following treatment [65], suggesting that the HIV cure observed in the "Berlin Patient" was at least in part a result of immune reconstitution with CD4+ T cells resistant to HIV infection. This has recently been suggested by a new similar case of HIV cure [66]. While these cases have encouraged HIV cure efforts, $CCR5^{\Delta 32/\Delta 32}$ individuals are rare, and

allogeneic stem cell transplant for the millions of individuals living with HIV is not feasible; however, this remarkable case has invigorated cure efforts and driven a massive amount of research into gene therapy to prevent expression of HIV co-receptors as a potential HIV cure.

In addition to gene therapy to make CD4+ T cells refractory to HIV infection, there are other strategies being developed to achieve HIV cure. The predominant strategy being investigated is the "shock and kill" approach, which aims to reactivate latent provirus, where upon cell death is caused by viral cytopathic effects, while HIV is unable to infect new cells due to suppressive ART [67]. This strategy relies on efficient stimulation of provirus from latent reservoir cells by latency reversal agents such as protein kinase C agonists or histone de-acetylase inhibitors, which to date have not been effective enough to significantly reduce the HIV latent reservoir, and sufficient ability of immune mediators such as HIV specific ADCC mediating broadly neutralizing antibodies to eliminate viral reservoirs has not been observed [68].

Recently, there has been evidence that early treatment may promote post-treatment control of HIV viremia. The case of the "Mississippi Baby", who was treated with ART from 30 hours post-delivery to 18 months of age and achieved ART free remission for 27 months [69] encouraged the use of early treatment to promote post treatment control. Furthermore, a few studies have suggested that limiting the size of the latent reservoir by treatment during acute HIV infection can lead to post-treatment control, characterized by undetectable viremia and a small latent reservoir that does not increase significantly following ART cessation [54, 70, 71]. However, early initiation of ART does not ensure post-treatment control as study participants initiating ART at equivalent times post infection did not always become post treatment controllers, suggesting alternative factors promoting post-treatment

control. HIV specific antibodies are an attractive immune control mechanism to limit the latent reservoir [72]. The RV144 vaccine trial observed that antibody-dependent cellular cytotoxicity (ADCC)-mediating antibodies were a correlate of protection [73, 74], while different studies have suggested that high levels of HIV specific ADCC antibodies limit the pathogenesis of HIV [75-77]; however, it is unknown if antibodies limit the seeding of the latent reservoir and promote post-treatment control. Understanding the factors that contribute to reservoir size and seeding may provide a framework for the path to post-treatment control.

Barring the identification of accurate biomarkers to detect post-treatment control while still on ART, evaluation HIV cure interventions will require treatment interruption. Assessing the impact of treatment interruption on the latent reservoir size is important as it may set back the progress made towards post treatment control. There have been few studies examining the impact of treatment interruption on the size of the HIV reservoir. In the CHER (Children with HIV Early Antiretroviral Therapy) study, infants treated within 3 months of birth underwent treatment interruption following ART for 40 or 96 weeks and were restarted on ART when their CD4 levels fell below 25%. They then measured the latent reservoir size of the infants randomized to treatment interruption once they were back on ART at the end of the study and compared them to infants who had waited till their CD4 levels dropped below 25% to start ART. The study observed that HIV proviral DNA in infants undergoing treatment interruption was not significantly different from infants in the deferred treatment group, suggesting that the limitation of the latent reservoir by early treatment was lost by treatment interruption [55]. Additionally, in a study of 3 infants who underwent treatment interruption for varying lengths of time, researchers observed a

substantial increase in reservoir size of one infant, experiencing a 52-fold increase in reservoir size with a 3 week transient treatment interruption, suggesting that recrudescent viremia following treatment interruption can generate significant increases in latent reservoir size [56]. However, studies of adults undergoing transient treatment interruptions displayed no significant increases in HIV reservoir size following treatment interruption, suggesting that the dynamics of reservoir seeding may be altered during recrudescent viremia [78, 79]. Understanding how treatment cessation influences reservoir size will be important to assess the risk and reward of potential curative strategies.

## 1.10 Dissertation summary

The goal of this thesis is to characterize the dynamics of the HIV DNA latent reservoir in individuals living in Kenya, where there is a high burden of HIV. Understanding when the HIV latent reservoir is seeded and what limits HIV latent reservoir size will inform future HIV cure studies seeking to eradicate or suppress the HIV latent reservoir. Chapter 2 describes the optimization and development of assays to quantify the HIV latent reservoir. Chapter 3 characterizes the HIV DNA reservoir size in early treated Kenyan infants and explores potential ways to limit reservoir seeding as well as identifying the impact of short treatment interruption. Chapter 4 characterizes HIV DNA reservoir seeding dynamics in a cohort of Kenyan women through phylogenetic comparison of sequences from pre-ART HIV plasm RNA and post-ART HIV DNA. Finally, a summary of my research and thoughts on future directions for HIV reservoir and cure studies is provided in Chapter 5.

# Chapter 2. Optimization and development of assays to quantitate the HIV latent reservoir

## 2.1 Introduction

ART can suppress HIV replication, however, a small population of quiescent cells containing integrated HIV DNA, but not producing virus, persists [80]. These cells are predominantly long lived CD4+ T cells that can stochastically produce virus upon cellular activation [81] and are known as the latent reservoir. The latent reservoir persists in part due to the long-lived nature of CD4+ T cells, but also because of clonal proliferation [36-38], necessitating life-long therapy. One strategy to eliminate the latent reservoir is to drive HIV out of latency using latency reversal agents that target the molecular mechanisms that promote persistence [82]. To assess the efficacy of such strategies, accurate reservoir quantitation methods are needed; and while there are currently both culture [47, 83] and PCR-based [50, 52, 84-88] assays that detect different species of the latent reservoir, no current assay accurately quantifies the replication-competent HIV reservoir [44-46].

The reason that PCR-based based assays fail to accurately quantitate the replication competent latent reservoir is because most of the latent reservoir is replication deficient, with up to 98% of latently infected cells containing replication deficient HIV DNA [46, 48, 51]. Even though PCR-based assays overestimate the size of the replication competent reservoir, they are attractive because they accurately quantitate viral nucleic acid from a small number of cells in which cell viability is questionable. This is especially relevant for use with infant cohorts, where

cell quantities are limited, as guidelines limit blood draws to 1-5% of total blood volume [89]; making it difficult to quantitate reservoir size by cell based assays such as the standard quantitative viral outgrowth assay (QVOA), which requires 120-180 mL of blood per assay [49].

The QVOA, which quantitates replication competent virus induced from latently infected CD4+ T cells, is the current gold standard assay [45]. While this assay detects only replication competent latent provirus it is labor intensive, costly, and requires large volumes of blood. Additionally, due to the stochastic nature of proviral reactivation [81], cellular activation stimuli used to induce latent provirus is inefficient, leading to a 60-fold underestimation of replication competent latent reservoir size [48]. Recently, a new assay, the TZA, was developed based on the QVOA that uses the TZM-bl reporter cell line, which contains the HIV receptor and co-receptor as well as an HIV-1 long terminal repeat promoter driven β-galactosidase gene, instead of donor PBMCs for viral outgrowth [90]. While the TZM-bl reporter cell line demonstrates increased sensitivity, destructive sampling is required for quantitation.

Here, for use during the outgrowth step of the QVOA similar to the TZA, we develop a reporter cell line that stably expresses high levels of CCR5 and CD4, and upon HIV infection produces GFP and secretes gaussia luciferase into the supernatant. Either of these markers can be used to identify latent provirus reactivation and quantify the latent reservoir. We demonstrate that this cell line can detect as little as 10 viruses in a sample which can be detected within 96 hours of infection, enabling more efficient detection of viral outgrowth without destructive sampling.

Additionally, to quantitate the latent reservoir using samples limited by cell quantity or viability we adapt and optimize a quantitative PCR based assay for use with droplet digital PCR (ddPCR). This assay detects cross-subtype HIV DNA by amplification of a conserved region of

the *pol* region. Here we demonstrate the sensitivity and accuracy of this assay, which can detect as few as 5 copies of HIV DNA in a million genomic equivalents extracted from frozen PBMCs, enabling accurate quantification from limited samples.

## 2.2 Materials and methods

***HIV DNA extraction from cell lines and clinical specimens***

*HIV DNA for ddPCR optimization:* Genomic DNA was extracted from the latently infected ACH-2 cell line (AIDS reagent program Catalog#: 349) using QIAamp DNA Giga Kit (Qiagen, Valencia, CA) and HIV DNA copy number was quantified by qPCR for the *pol* gene [91].

*Genomic DNA for ddPCR optimization:* Genomic DNA was extracted from the CEM.NKR cell line (AIDS reagent program Catalog#: 458) using QIAamp DNA Midi Kit (Qiagen, Valencia, CA) and genomic equivalents was quantified by Nanodrop spectrophotometry.

*Clinical samples for QVOA optimization*: Frozen PBMC samples from HIV-infected patients in Seattle, Washington infected with subtype-B strains was obtained from the Center for AIDS Research [92] repository. PBMCs were collected from viremic and ART suppressed individuals. PBMCs were thawed and DNA was extracted from peripheral blood mononuclear cells (PBMCs) using QIAamp DNA Midi Kit (Qiagen, Valencia, CA).

***ddPCR to quantify HIV and human genomic DNA***

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) or indicated cell lines using QIAamp DNA Midi Kit (Qiagen, Valencia, CA). Cellular DNA was quantified using RPP30 ddPCR assay (Catalog #: 100-31243, Bio-Rad, Hercules, CA). Prior to a one hour digestion

with BSAJI(NEB), extracted DNA was heated to 95˚C for 10 minutes and placed on ice for 5 minutes as previously described [86]. HIV DNA was quantified by in-house cross-subtype *pol* polymerase chain reaction (PCR) [93] modified for ddPCR. In brief, each reaction consisted of a 20 µL solution containing 10 µL ddPCR Probe Supermix no dUTP (Catalog #:1863024, Bio-Rad, Hercules, CA), 900 nM pol4ext primer (CTA CTG CCC CTT CAC CTT TCC), 900 nM pol15ext primer (TAC AGT GCA GGG GAA AGA ATA ATA G), 250 nM polp1 probe (6FAM TTT CGG GTT TAT TAC AGG GAC AGC AG TAMRA), and extracted DNA. Each sample was combined with 70 µl of oil (Catalog #:1863005, Bio-Rad, Hercules, CA) in a ddPCR cartridge (Catalog #:1864008, Bio-Rad, Hercules, CA) to generate a 40µl emulsion containing nanoliter droplets. Droplets were transferred to a 96 well plate and placed in a thermal cycler with the following cycling conditions: 10 minutes at 95˚C, 40 cycles each consisting of a 30 second denaturation at 94˚C followed by a 60˚C extension for 60 seconds, and a final 10 minutes at 98˚C. After cycling, samples were analyzed on the Bio-Rad QX200.

***Cells and viruses***

Q23-BSM-BG505 (Gift from Daryl Humes), is a replication competent HIV variant that was generated by inserting a BG505 *env* gene variant into the Q23 genome. Q23-BSM-BG505 virus was titered on TZM-bl cells. The following cell lines were obtained from the NIH AIDS Reagent Program: TZM-bl (catalog number: 8129), ACH-2 (catalog number: 349), CEM.NKr-CCR5 (catalog number: 4376), MOLT-4-CCR5 (catalog number: 4984), and CF2TH SynCCR5 (catalog number: 4662). Supt1-R5 (SUPHUR5 were a gift from James Hoxie), JC.53 [94], and 293t-hCD4-hCCR5 (Generated by Jeremy Roop) cells were generously donated. Cells were cultured according to the media described in Table 2.1.

**Table 2.1. Cell lines and media used to generate QVOA indicator cell lines.**

| Cell Line | Media | Selective Antibiotic |
|---|---|---|
| Molt-4 CCR5 | RPMI/ 10% FBS/ 1% L-glutamine/ 1% PSF | 1mg/ml Geneticin |
| SUPT1-CCR5 | RPMI/ 10% FBS/ 1% L-glutamine/ 1% PSF | None |
| CEM-NKR-CCR5 | RPMI/ 10% FBS/ 1% L-glutamine/ 1% PSF | None |
| JC.53 and TZM-bl | DMEM/ 10% FBS/ 1% L-glutamine/ 1% PSF | None |
| 293t HuCD4/HuCCR5 | DMEM/ 10% FBS/ 1% L-glutamine/ 1% PSF | None |
| CF2TH SynCCR5 | DMEM/ 10% FBS/ 1% L-glutamine/ 1% PSF | 400µg/ml Geneticin, 2µg/ml Puromycin |

***Generation of reporter cell lines that express gaussia luciferase upon HIV infection***

When integrated into genomic DNA, the pNL-GFP-RRE(SA) vector leverages HIV encoded transactivating response element, the rev response element (RRE), and HIV splice sites to produce the reporter gene only upon HIV infection of the transduced cell line [95]. In order to create a construct that, when integrated into genomic DNA, would produce guassia luciferase following HIV infection, we introduced the *gaussia luciferase* gene in the multiple cloning site upstream of the GFP reporter. In short, the *gaussia luciferase* gene was amplified from the pCMV-Gaussia-Dura-Luc vector (ThermoFisher) using primers (XmaI_pCMVGluc_646F- ATC CCG GGA TGG GAG TCA AAG TTC TGT TTG C and SphI_pCMVpolya_1336R- ATG CAT GCA TGC AAT TTC CTC ATT TTA TTA GGA) with

restriction sites engineered onto the ends. The PCR product and pNL-GFP-RRE(SA) were digested with SMA1 and SPH1 and subsequently ligated to create pNL-gluc-GFP-RRE(SA) (Figure 2.1).

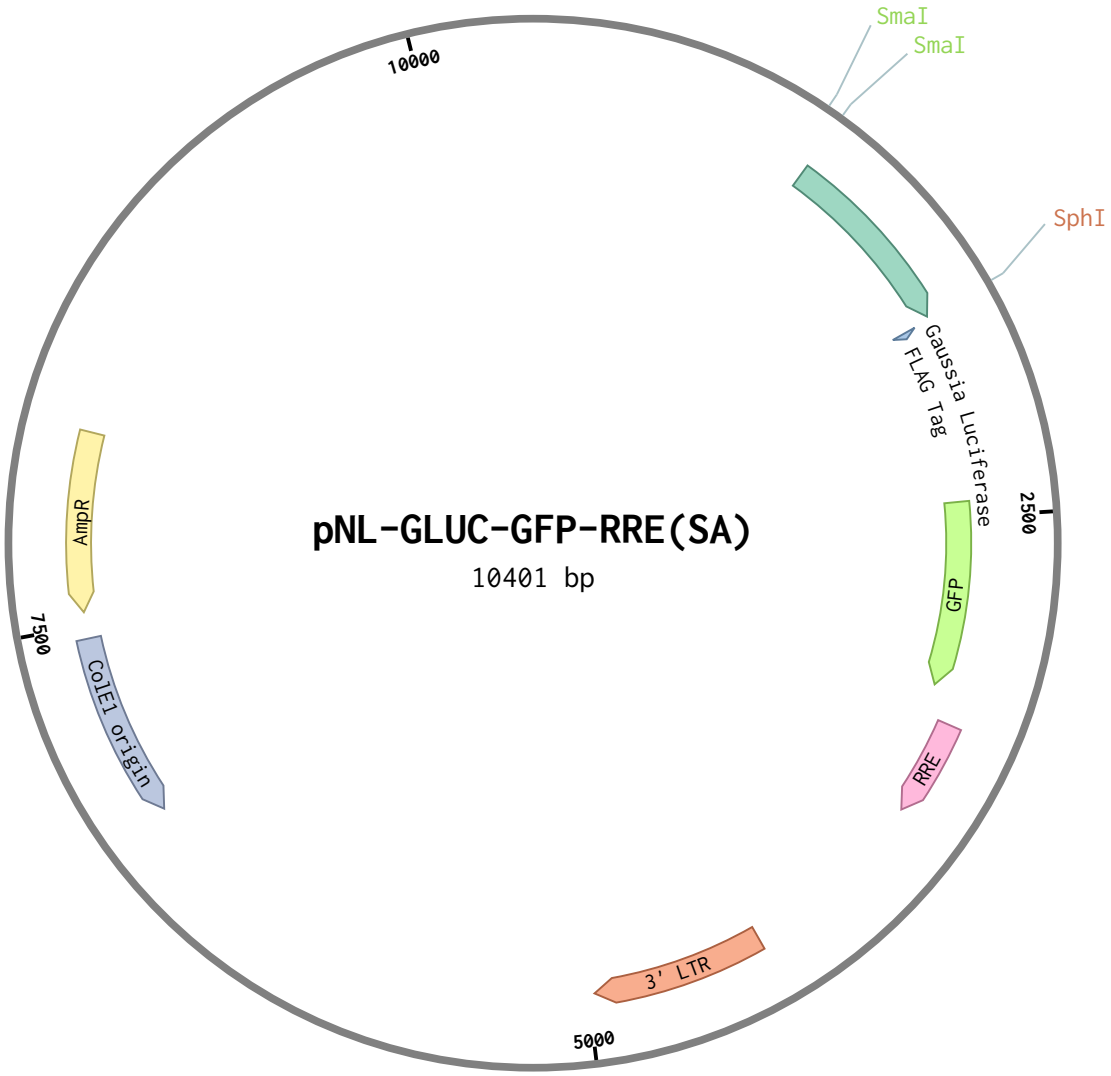


**Figure 2.1. Plasmid map of pNL-gluc-GFP-RRE(SA).**

To create generate retroviral virus-like particles to stably transduce cell lines, we used FuGene 6 (Roche) to cotransfect pNL-gluc-GFP-RRE(SA), psPAX2, and pMD.G in 293t cells at a 1:1:0.5 ratio to produce viral like particles pseudotyped with the VSV-G envelope. 48 hours after transfection, supernatant was harvested and concentrated using a 100k amicon concentrator. 20µl

of concentrated viral like particles was added to 250,000 cells of each of the 6 cell lines in a 6 well plate and plates were spinoculated for 90 minutes. Media was then replaced and cells were left to recover. Bulk cultures were single cell sorted into two 96 well plates and grown until confluent. Cell growth was assessed by microscopy and wells with evidence of cell growth were assayed (Pierce Gaussia Luciferase Glow Kit, Catalog #: 16161) for luciferase induction 24, 48, and 96 hours following infection with Q23-BSM-BG505. Wells with luciferase activity were expanded and cryogenically preserved. One Supt1-R5 clone was chosen to explore for potential use as an outgrowth indicator cell line in the QVOA.

***Infection of TZM-bl and Supt1-R5 indicator cells with Q23-BSM-BG505***

TZM-bl or Supt1-R5 indicator cells were plated in a 96 well plate at a concentration of 50,000 cells in 200µl with 3µg/mL DEAE Dextran. Two fold dilutions of HIV Q23-BSM-BG505 (TZM-bl titer: 2.77e6 infectious units per mL) were made in RPMI and virus was added to each well. 48 hours after infection TZM-bl infected cells were screened with Gal-Screen™ β-Galactosidase Reporter Gene Assay System for Mammalian Cells (ThermoFisher Scientific), and Supt1-R5 indicator cells were screened with Pierce™ Gaussia Luciferase Glow Assay Kit (ThermoFisher Scientific) according to their respective protocols.

***Allogeneic PBMC isolation***

Allogeneic donor PBMCs were isolated from Pall filters obtained from Bloodworks Northwest the day of the assay. PBMCs were irradiated with 5000R in a cesium source irradiator. Following irradiation, CD8+ T cells were depleted using Dynabeads (ThermoFisher Scientific). CD8

depleted PBMCs were counted and stored in PBMC culture medium (RPMI/10% FBS/1% L-Glutamine/1% P/S/F) at 37˚C with 5% CO2 until added to patient PBMCs where indicated.

***Quantitative viral outgrowth assay (QVOA) protocol***

QVOA was performed as described by Laird et al. [96] with the following modifications: ~5e6 patient PBMCs were thawed and re-suspended in 9mL RPMI containing benzonase. Cells were centrifuged at 800g for 10 minutes, re-suspended in 1mL of PBMC culture medium and placed in a T-25 flask. The flask was incubated for 2 hours at 37˚C with 5% CO2 to remove monocytes. Following incubation cells were centrifuged at 800g for 10 minutes and re-suspended in 1 mL of PBS with 2% FBS and 1mM EDTA. CD4+ T cells were isolated using a Stemcell EasySep™ CD4 isolation kit (Catalog#: 17952) and re-suspended in 1mL of PBMC culture medium containing 100 U/mL IL-2. Cells were counted and split across wells containing ~5e3 cells into replicates in either a 24 or 48 well plate. Irradiated allogeneic PBMCs were added in 10-fold excess. Finally, cells were stimulated by the addition of 1μg/mL PHA. 12 hours later 500μl of PBMC culture medium was added to the 48 well plates, and 1 mL to the 24 well plates. After 24 hours 2/3 of the media was removed to prevent PHA toxicity. Either 250,000 or 100,000 Supt1-R5 indicator cells (Clone 5) in PBMC culture medium with 10u/mL IL-2 were added in volumes of 1.5 mL or 1 mL to the 24 or 48 well plates respectively and incubated at 37˚C with 5% CO2. Wells were assayed for outgrowth by Pierce™ Gaussia Luciferase Glow Assay Kit (ThermoFisher Scientific) following the standard protocol.

## 2.3 Results

***Optimization of pol droplet digital PCR (ddPCR) to measure total HIV DNA in the latent reservoir***

We used previously validated cross-subtype primers and probe from qPCR targeting the *pol* gene (See Methods)[91] to optimize a ddPCR assay to quantitate the HIV DNA latent reservoir. ddPCR has been shown to be more accurate and precise than qPCR without the need for a standard curve [86]. In order to optimize sensitivity with the ddPCR total HIV DNA assay, well quantified DNA extracted from the latently infected ACH-2 cell line (1 HIV integration event per cell[97]) was mixed with known quantities of genomic DNA from the CEM.NKR cell line to create controls that contained 10 and 100 HIV DNA copies. Based on previous data from Strain et al, we tested increasing amounts of human genomic DNA added to ACH 2 cell DNA to determine concentrations that inhibit our *pol* ddPCR and observed that at concentrations >2μg per assay a greater than 2-fold reduction in HIV DNA quantitation occurred (Figure 2.2). Up until that amount, which corresponds to approximately 320,000 cells, there was no inhibition. Because the average treated individual contains between 100 and 1000 copies of HIV DNA per million PBMCs [98], our assay would be able to quantify HIV DNA in most samples.

To identify the limit of detection for the *pol* ddPCR assay we repetitively measured the number of false positive droplets in samples containing CEM.NKR genomic DNA at a concentration of $10^5$ cells/assay (~0.66μg). Across 25 replicates, we observed 3 false positive droplets, corresponding to a false positive rate of 1.4copies/$10^6$ cells and a limit of blank (LOB) of 1.11 [99]. We next determined the limit of detection (LOD) using guidelines from Armbruster et al. by assaying controls that were well quantified by qPCR at 2 copies of HIV DNA/sample [99]. Using the equation

LOD = LOB + 1.645(SD(Low Concentration Sample) we determined a limit of detection of 5 copies per assay. Furthermore, we created controls containing 0.1, 1, 10, and 100 HIV copies in $10^5$ cells by diluting well quantified ACH-2 DNA into CEM.NKR DNA quantified by spectrophotometry. Ten replicates of each control were assayed for *pol* HIV DNA. We detected HIV DNA in 2/10, 4/10, 10/10, and 10/10 samples from the 0.1, 1, 10, and 100 copy samples respectively. Assaying a total of $10^6$ cells across 10 replicates we show that our HIV *pol* assay accurately quantifies as few as 1 copies of HIV DNA in a genomic background of $10^5$ cell equivalents (Figure 2.3).

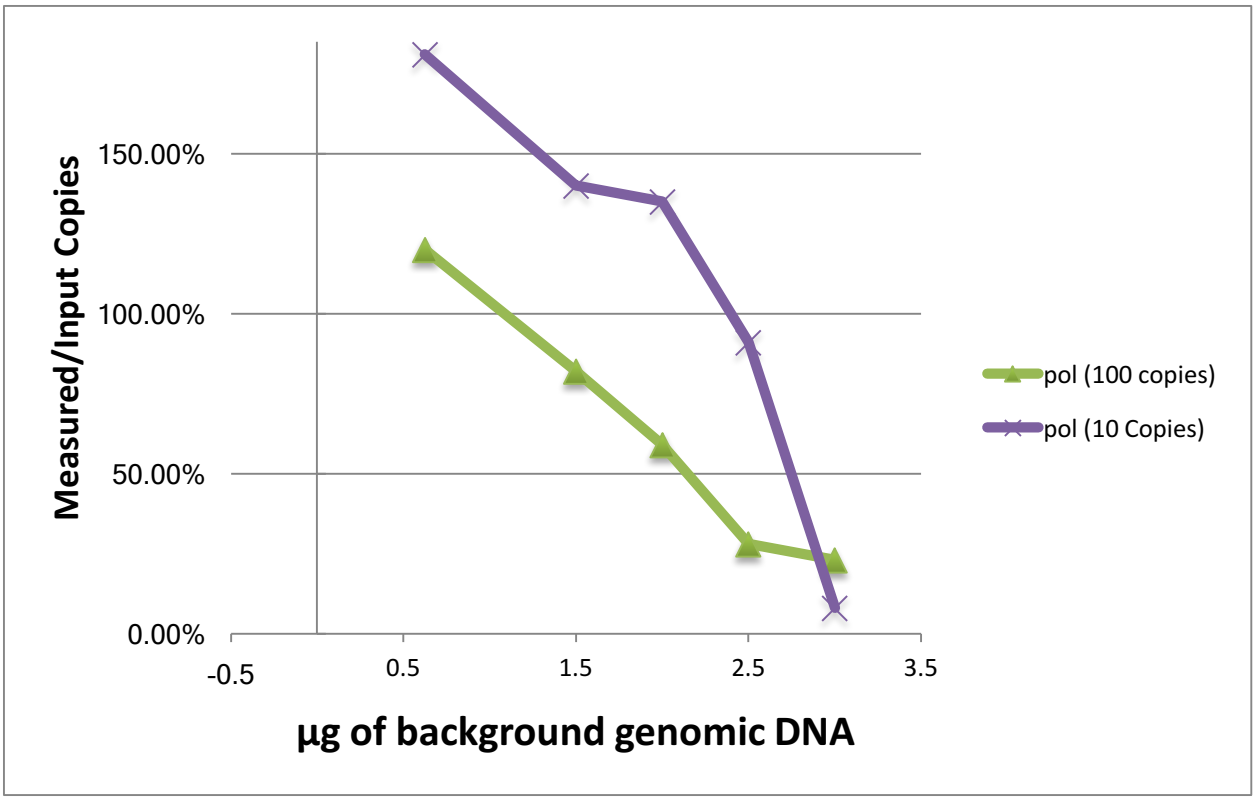


**Figure 2.2. HIV DNA quantified in the presence of genomic DNA.**
HIV DNA extracted from ACH-2 Cells was diluted in genomic DNA extracted from CEM.nkr cells and ddPCR for pol HIV DNA was assayed. At 1.5µg of background genomic DNA, HIV DNA quantitation is inhibited.

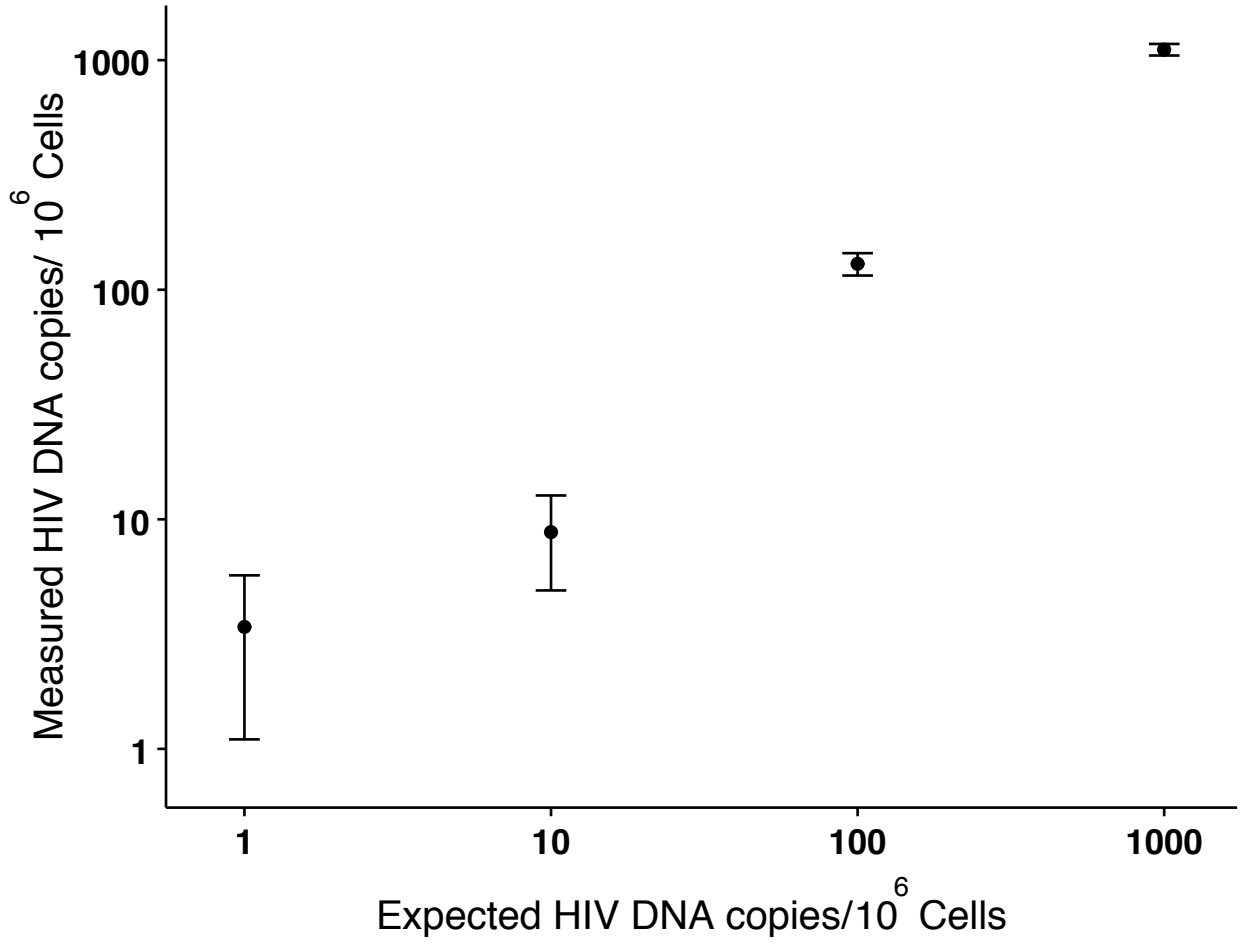


**Figure 2.3. Comparison of observed versus expected total HIV DNA copies/$10^6$ cells by pol ddPCR on well-quantified and previously validated controls.**
ACH2 cells with a single copy of HIV DNA serially diluted into HIV-negative CEM cells. Ten replicates of each HIV DNA level control (0.1, 1, 10, and 100 HIV copies in $10^5$ cells) were performed on DNA from $10^5$ total cells per replicate, resulting in $10^6$ total cells at each HIV DNA level tested: 1, 10, 100 and 1000 HIV copies per $10^6$ cells. LOD for the pol ddPCR assay was defined as 5 copies of HIV DNA/$10^6$ cells. Error bars represent the standard error of the mean.

***Development of a cell line for QVOA outgrowth and latent provirus reactivation detection***

We developed an HIV sensitive cell line that contains a tat and rev responsive gaussia luciferase reporter gene using the pNL-GLUC-GFP-RRE(SA) vector (described in methods) for QVOA outgrowth and latent provirus reactivation detection. Six cell lines were chosen as candidates for transduction with pNL-GLUC-GFP-RRE(SA) based on the fact that they either expressed high levels of CD4 and CCR5 (JC.53, CEM.NKr-CCR5, 293t-hCD4-hCCR5 -Gift from Jeremy Roop, and CF2TH

SynCCR5)[94, 100, 101], or had been previously used in QVOA's (Supt1-R5 and MOLT-4-CCR5) [102, 103]. Reporter cell lines were generated and bulk transductions were assayed for luciferase production following infection with HIV. The CEM.NKr-CCR5 and Supt1-R5 bulk transductions had the highest induction of luciferase activity following 48 hour infection with Q23.bsm.BG505 at a multiplicity of infection of 1 (37 and 40 fold over background respectively); and we decided to move forward with the transduced Supt1-R5 cell line based on the fact that it had been used previously in a QVOA [103]. Clonal cell lines were isolated by single cell sorting bulk transduced cells and assaying for luciferase production following infection with HIV. Clonal cell lines with the largest induction of gaussia luciferase following infection with HIV were chosen as potential candidates for QVOA outgrowth and reactivation reporters. The Supt1-R5 cell line was shown to have high expression levels of both CD4 and CCR5 expressed on the cell surface [104] and is cultured in the same medium as primary CD4+ T cells in the QVOA protocol, suggesting it would be easily adaptable to the QVOA assay. Clone 5, a Supt1-R5 clonal reporter cell line had high levels of luciferase induction following HIV infection was chosen as a potential QVOA outgrowth and reporter candidate (Figure 2.4).

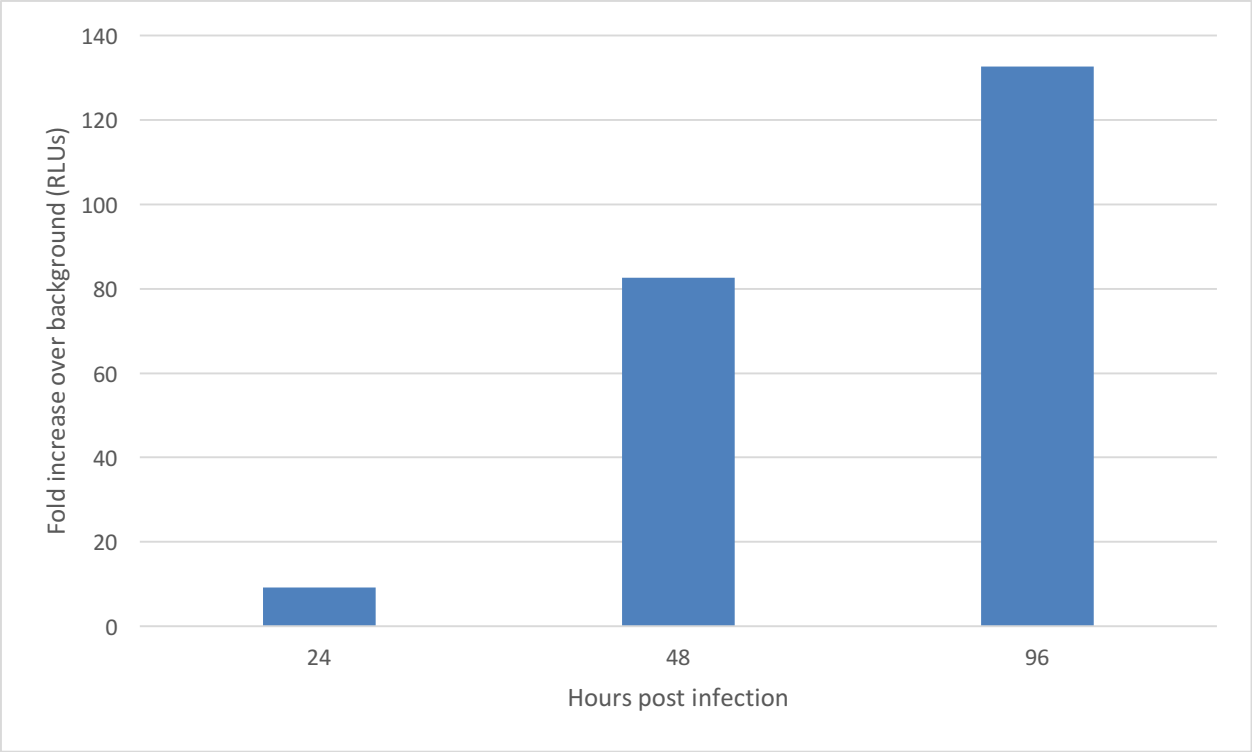


**Figure 2.4. Time course infection of a Supt1-R5 indicator cell line clone.**
Fold change of RLUs over background following Supt1-R5 clone (80,000 cells) infection with Q23.bsm.BG505 (MOI: 0.4) after 24, 48, and 96 hours.

***Sensitivity of a clonal Supt1-R5 reporter cell line to HIV infection***

QVOA sensitivity is reliant upon detection of virus produced from one latently reactivated cell. Because there are around $10^4$ viral particles produced from one cell [105], we wanted to validate that clone 5 could identify quantities of virus one would expect to be produced from reactivation of one latently infected cell. To determine the sensitivity of clone 5, we infected it with the replication competent HIV strain Q23.bsm.BG505 at a range of multiplicity of infections (MOIs) between 2e-5 and 0.3, determined by tittering on TZM-bl cells, and compared to TZM-bl cells infected with the same range of MOIs. After 48 hours of infection, luciferase values greater than a two-fold increase over background in clone 5 was apparent at an MOI of 8.7e-3(433 viral particles) compared to an MOI of 6.8e-5 (3 viral particles) in the TZM-bl cells (Figure 2.5).

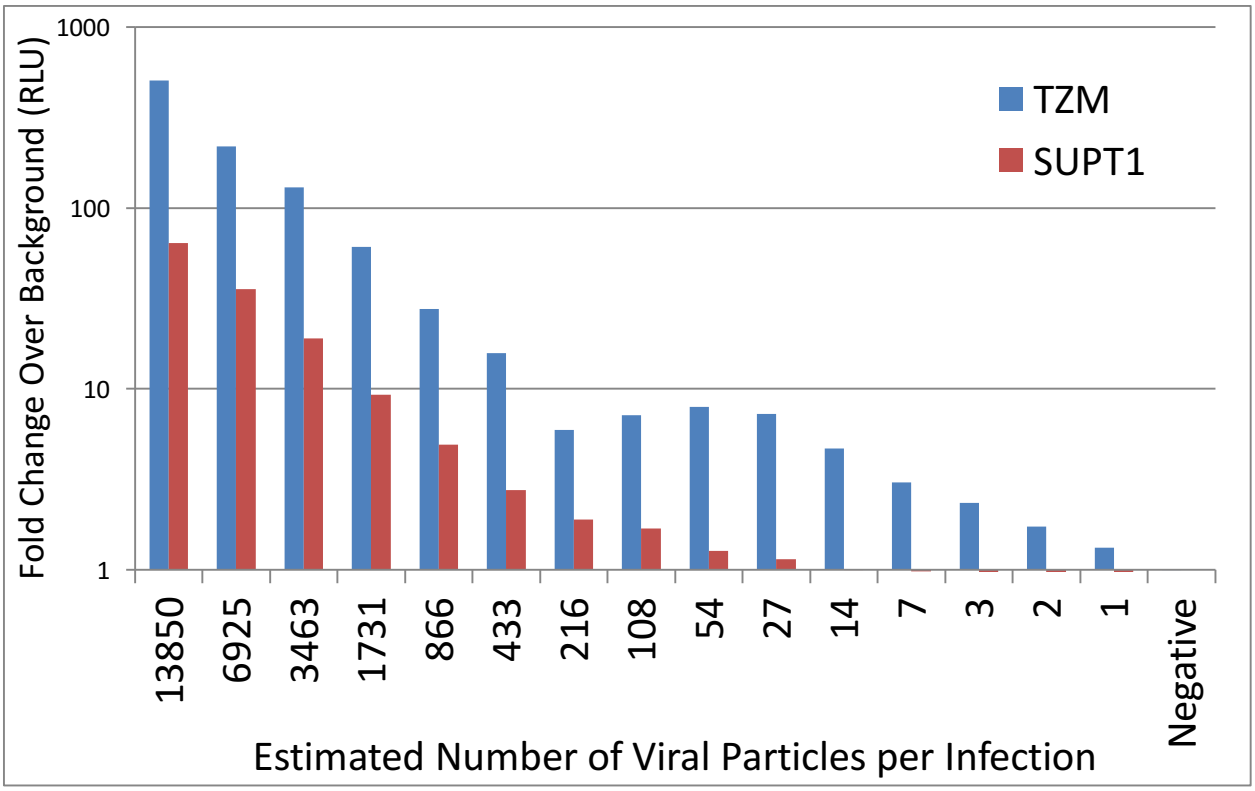


**Figure 2.5. Infection sensitivity comparison of Supt1-R5 indicator cell line clone 5 with TZM-bl cells after 48 hours.**
TZM-bl and Supt1-R5 indicator cell line clone 5 were infected with serially two-fold diluted Q23.bsm.BG505 virus and assayed for infection by luciferase activity 48 hours after infection. >2 fold over background luciferase activity was observed at an infection with 3 viral particles for the TZM-bl cell line and 433 particles for Supt1-R5 indicator cell line clone 5.

However, when SUPT1-R5 indicator cell line clone 5 was cultured for an additional 7 days, we observed luciferase values greater than a two fold increase over background in cells infected with an MOI of 3.4e-5 (2 viral particles), suggesting that our engineered clonal cell line was sensitive enough to detect as few as 2 viral particles following expansion of virus in outgrowth

cells (Figure 2.6).

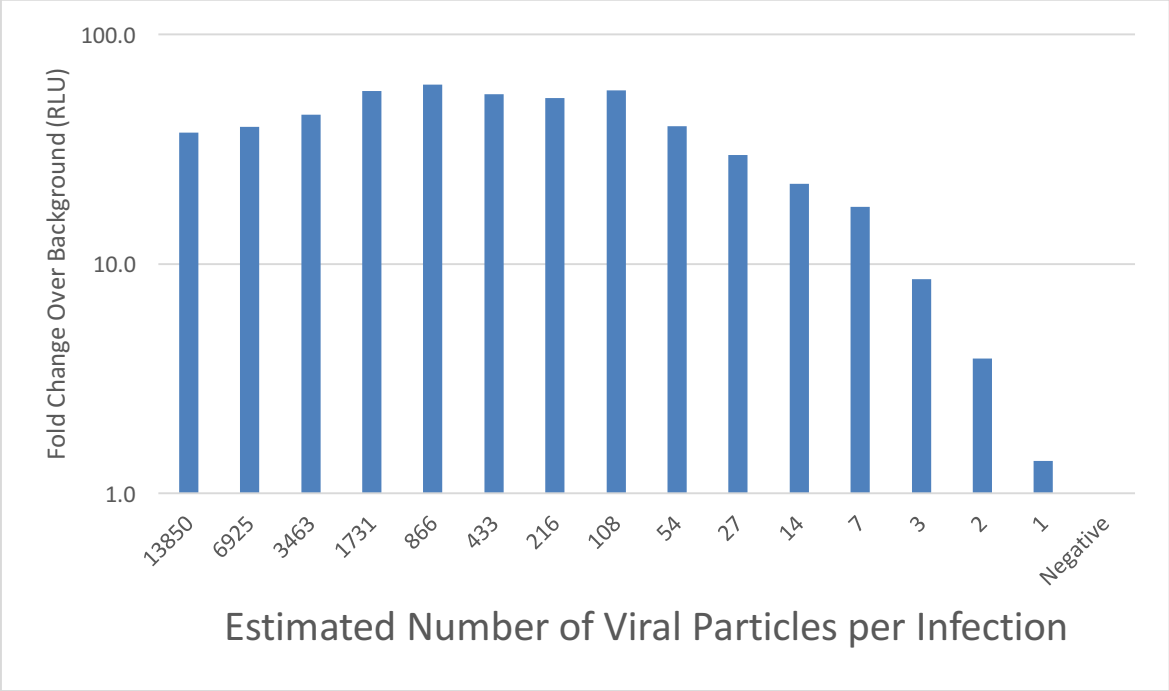


**Figure 2.6. Infection sensitivity of Supt1-R5 indicator cell line clone 5 after 9 days.** Clone 5 was infected with serially two-fold diluted Q23.bsm.BG505 virus and assayed for infection by luciferase activity 9 days after infection. >2 fold over background luciferase activity was observed at an infection with 2 viral particles for Clone 5.

***Testing the QVOA gaussia luciferase assay on HIV-infected patient PBMCs***

To test our modified QVOA assay we obtained duplicate PBMC samples from 4 HIV-infected subjects from the Center for AIDS Research repository. One vial of each subject's PBMCs was used for HIV DNA quantitation while the other vial was screened for viral outgrowth using a modified viral outgrowth assay (see methods). To identify whether we could detect virus from reactivated latently infected cells, we chose three donors (1136, 1882, and 2111) on ART; and to identify whether we could detect virus from untreated infection, we chose one donor (1842) not on ART as a positive control. HIV DNA ranged from 31-124 c/$10^6$ PBMCs in the treated donors and was highest (7,866 c/$10^6$ PBMCs) in the untreated donor (Table 2.2). Based on the fact that ~98% of HIV DNA

is estimated to be replication deficient and our samples came from 5e6 total PBMCs, we expected 3 to 12 latently infected cells harboring replication competent per ART suppressed donor. We isolated ~220,000-480,000 CD4+ T cells per donor. To give ourselves the best chance at detecting latently infected cells we chose to forgo limiting dilution in favor of conservatively spreading out our CD4+ T cells at a concentration of 10,000 CD4+ T cells per well. Viral outgrowth was identified by luminescence 6 wells at day 11 and in 10 wells at day 33 for donor 1842 who had a high viral load and the highest HIV DNA quantity; in contrast, viral outgrowth was not observed by luminescence in any of the virally suppressed donors (2111, 1136, and 1882) (Figure 2.7).

**Table 2.2. HIV DNA and viral load of CFAR repository PBMC samples used to detect outgrowth.**

| Donor ID | Cells/µl | HIV DNA copies/µl | HIV DNA copies/$10^6$ PBMCs | On ART? | Closest viral load to specimen (c/mL) |
|---|---|---|---|---|---|
| 1842 | 8730 | 70.9 | 7,866 | No | 159,300 |
| 1136 | 14680 | 1.4 | 94 | Yes | Undetectable |
| 1882 | 8620 | 0.9 | 124 | Yes | Undetectable |
| 2111 | 17280 | 1.3 | 31 | Yes | Undetectable |

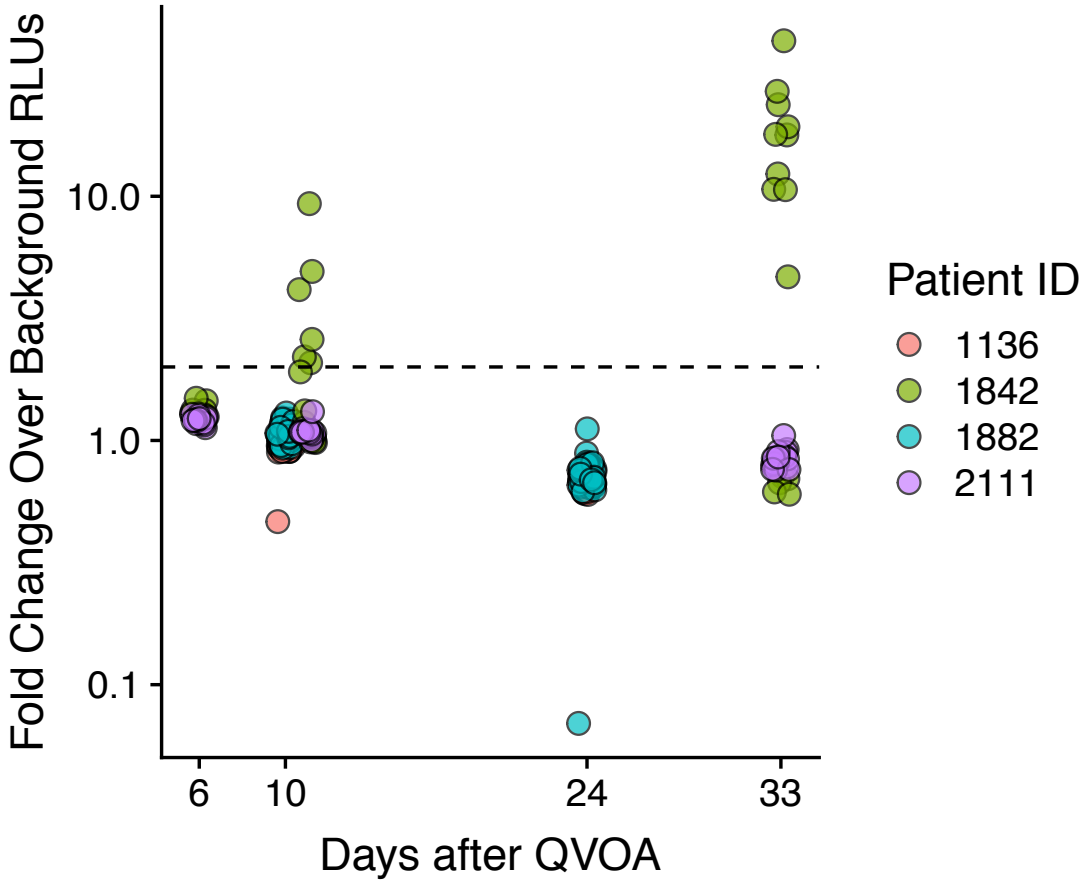


**Figure 2.7. Sensitivity of Supt1-R5 indicator cell line clone 5 to reactivated HIV from latently infected donor CD4+ T cells.**
Latently infected patient derived CD4+ T cells were stimulated with 1µg/mL PHA, added to donor PBMCs, and co-cultured with the Clone 5. Supernatant was assayed for luciferase activity. Presence of replication competent HIV was identified by RLUs >2 fold over background in Patient ID 1842.

## 2.4 Discussion

Accurate, low cost methods to quantify the HIV latent reservoir are needed to understand the establishment, seeding dynamics, and decay of the HIV reservoir as well as to assess the efficacy of novel HIV cure strategies. Here we present two methods, one to quantify total HIV DNA (including both replication competent and replication deficient provirus) and one to quantify only replication competent HIV. While HIV DNA quantitation by ddPCR is a standard method in the HIV

latent reservoir field, our method leveraged previous studies of primers that allowed detection across subtypes common in Africa. We also created a novel cell line that can potentially be used to improve the read out for the quantitative viral outgrowth assay to reduce assay time, cost, and false negative results.

It has been well established that ddPCR protocols for HIV DNA have higher precision than similar qPCR protocols [86], which is important since HIV latent reservoir cells can be extremely rare, with as few as 1 copy of HIV DNA per million resting CD4+ T cells. In this study we show that our previously published qPCR primers and protocol [93] for the *pol* region of HIV DNA can be adapted for the ddPCR system. Furthermore, our ddPCR assay detected as little as 1 copy of HIV DNA in a background of 2.5e4 genome equivalents, and was accurate when as much as 1µg of DNA was in a sample.

While our pol ddPCR assay can accurately and reliably detect small quantities of HIV DNA in a dense background of genomic DNA, it cannot distinguish between replication deficient and replication competent provirus, leading to an overestimation of the replication competent HIV latent reservoir. Additionally, replication deficient proviruses that include mutations or deletions within the primer/probe binding sites will be missed by the ddPCR assay, leading to an underestimation of the total HIV DNA. It is still unclear whether the replication deficient reservoir is important in disease pathogenesis, but studies are ongoing [106].

In the context of ART discontinuation, the replication competent latent reservoir is the source of viral rebound from an HIV infected, aviremic individual [27] and the major barrier to an HIV cure. Sensitive assays are needed to identify whether HIV cure strategies targeting the latent reservoir have succeeded in its elimination or reduction. Here, we created a cell line to detect the reactivation of replication competent provirus from latently infected cells to be used instead of

donor CD4+ T cells [107] or TZM-bl cells [90] in the QVOA. We show that this Supt1-R5 indicator cell line is capable of secreting high concentrations of gaussia luciferase into the supernatant upon infection with HIV, and furthermore, can detect as little as 2 replication competent HIV particles after 9 days of culture. Since the quantity of viral particles produced from one infected CD4+ T cell is expected to be in the range of $10^4$ [105], we believe our cell line is sensitive enough to detect one reactivation event in a well. However, our studies did not directly address this and further studies are needed to test the use of these cells for detecting replication-competent virus from the latent reservoir.

When we reactivated CD4+ T cells from patient samples we only observed high levels of luciferase in the PBMCs from an untreated individual with high levels of viremia. Virus replication was not detected in any of the PBMCs from ART-suppressed patients. This could be due to several reasons. The samples that we obtained were limited in cell number (5e6 PBMCs) and based on our HIV DNA quantitation we would expect a maximum of 620 cells containing total HIV DNA (both replication deficient and replication competent) from Donor ID 1882. Given that there have been estimates that ~93-98% of HIV DNA detected is replication deficient [51], we might expect that at most our sample contained 12-43 replication competent proviruses. Furthermore, Ho et al. has shown that not all replication competent proviruses are reactivated with a single round of stimulation, suggesting that multiple rounds of activation are needed to accurately quantify the replication competent provirus population [48]. Thus, it is perhaps not surprising that we did not observe viral outgrowth in our limited sample set.

In addition to detecting HIV with high sensitivity, our strategy requires no addition of donor PBMCs for outgrowth and allows for repetitive sampling of outgrowth cultures, which is not available with the TZA assay [90]. The outgrowth from latently infected cells still relies on efficient

activation of latent provirus, and thus does not improve upon other viral outgrowth strategies in that manner. However, our cell line provides a novel, cost effective method for screening for HIV infection and thus has the potential to be an important tool in screening latency reversal agents and developing more accurate QVOAs.

# Chapter 3. The impact of early treatment, antibody dependent cellular cytotoxicity, and short treatment interruption on the HIV DNA reservoir in Kenyan infants

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## 3.1 Introduction

During acute HIV infection, a reservoir of long-lived infected cells is established that persists during antiretroviral treatment (ART) and causes viral rebound upon ART cessation [34, 57]. Experimental non-human primate models show that this reservoir is generated within days of infection [53]. Initiating ART early in HIV infection may limit reservoir size, increase time to viral rebound upon treatment cessation, and increase likelihood of post-treatment viral control - but does not ensure remission [54, 57, 79, 108]. In adults, initiation of ART during acute HIV infection has been shown to limit HIV DNA reservoir size, suggesting that reservoir size is directly associated with early ART initiation [109]. Furthermore, in case reports, HIV-infected infants with early ART had long periods of remission [69, 70] encouraging efforts to identify interventions that augment early ART to promote post-treatment control in pediatric populations.

While levels of autologous neutralizing antibodies have not been shown to be associated with reservoir size[110], antibody effector functions, such as antibody-dependent cellular cytotoxicity [77], may kill latently infected cells and reduce the size of the latent reservoir [111]. Moreover, ADCC mediating antibodies were a correlate of protection in the RV144 vaccine trial [74], and levels of ADCC antibodies were correlated with disease progression [75]. Furthermore, studies from our lab have suggested that in the context of mother to child transmission ADCC

activity is associated with improved infant survival, suggesting that passively acquired ADCC is important in limiting HIV pathogenesis [112]. Studies are needed to identify the role of ADCC in latent reservoir seeding and their potential as a therapeutic to achieve HIV cure.

Evaluation of the use of antibodies and other novel HIV cure strategies will require treatment interruption (TI). Thus, it is important to understand whether short TI leads to sustained increases in latently infected cells in the HIV reservoir. Few studies have measured the impact of TI on HIV reservoir. In adults, TI has been associated with initially increased HIV DNA levels, which return to pre-TI levels after >6 months of ART resumption, suggesting TI may not cause a lasting increase in HIV-infected cell reservoirs [78, 79]. In contrast, a preliminary report from the Children with HIV Early Antiretroviral Therapy trial (CHER) showed sustained increased HIV DNA levels in 17 infants 2-3 years following a median 11 month TI [55], and a smaller study showed similar results [56].

In order to identify the roles of both early treatment and ADCC activity in the seeding of the HIV DNA reservoir, we quantified blood HIV DNA reservoir levels in a cohort of Kenyan infants two years after ART initiation. We compared early vs late treatment and pre-ART ADCC activity with HIV DNA reservoir size. In addition, to understand the impact of shorter TI in infants, we quantified blood HIV DNA reservoir levels of infants who were randomized to continued ART versus three month long TI, eighteen months following randomization. Finally, to identify whether ADCC activity during TI limited reseeding of the latent reservoir during TI, we measured ADCC activity three months following randomization to TI versus continued therapy, and assessed the relationship between ADCC levels and change in reservoir size following TI.

## 3.2 Materials and methods

***Study population***

The Optimizing Pediatric HAART trial (NCT00428116) was a randomized controlled trial in which, following 24 months of continuous ART (median age at initiation: 5 months), children were randomized to continued ART or TI [113]. Blood was collected at 3-month intervals for CD4 measurements in real time and stored for HIV viral load (performed retrospectively). ART restart criteria were CD4% <20–25%, or >one-third decrease from peak CD4, more advanced WHO stage, or weight-for-age decrease as described [113]. After ART restart there was no further interruption. Children were excluded from this sub-study if HIV RNA was ≥1000 copies/mL during 6 months prior to the HIV DNA measurements at 24months post-ART initiation (time of randomization) or 42 months post-ART initiation (18 months following randomization); or if samples were not available.

***Laboratory methods***

Plasma HIV RNA was quantified using the Gen-Probe HIV-1 RNA assay (Gen Probe, San Diego, California) with a limit of detection (LOD) of 2.18 $\log_{10}$ copies/ml. DNA was extracted from PBMCs using QIAamp DNA (Qiagen, Valencia, CA). Cellular DNA was quantified using RPP30 ddPCR assay (Bio-Rad, Hercules, CA). HIV DNA was quantified in duplicate by in-house cross-subtype *pol* PCR [93] modified for ddPCR. If results were below the LOD or >2-fold discordant between duplicates, additional replicates were performed until $>10^5$ cells were tested. HIV DNA was normalized to RPP30 and HIV DNA copies/$10^6$ PBMCs reported. ADCC activity was measured by the Rapid Fluorometric ADCC (RF-ADCC) assay [114] as previously described

[112] using HIV gp120 antigen BL035. All samples were assayed for ADCC at a 1:1000 dilution in two independent experiments in duplicate.in duplicate? Technical replicate?

***Statistical analysis***

Analysis was performed using R (version 3.4). HIV DNA reservoir size between infants starting ART prior to 3 months to those starting ART after 3 months was compared using the Wilcoxon rank-sum test. HIV DNA fold change between continued and TI arms was also compared using the Wilcoxon rank-sum test. Correlation between post-randomization peak HIV RNA and HIV DNA fold change as well as between post randomization ADCC activity and HIV DNA fold change was determined using Spearman's Rank-Order Correlation.

## 3.3 Results

***Cohort characteristics during initial 24 months of ART***

In order to identify correlates of HIV DNA reservoir size, 39 infants from the OPH cohort who maintained viral suppression throughout 2 years on ART, defined as having at most 1 viral load above 1000 copies/mL were included in our analysis comparing HIV DNA reservoir size to ART initiation age (range 2.5-11.3 months). Furthermore, to identify whether ADCC activity at ART initiation correlated with HIV DNA reservoir size, 25 of the 39 infants who had plasma samples for available at ART initiation (range 2.5-11.3 months) were included in our analysis comparing ADCC activity to HIV DNA reservoir size. At ART initiation, median age of the 39 infants was 4.8 months (IQR 4.3-7), median CD4% was 22% (IQR 15-25) and median viral load was 6.5 $\log_{10}$ copies/mL (IQR 6.1-7.0) (Table 3.1). Initial ART was NNRTI-based for 27 infants

and PI-based for 12 infants. Twenty-four months following ART initiation (the time of randomization) 9 children were still on NNRTI-based ART and 30 were taking PI-based ART; median CD4% had risen to 35% (IQR 32-40) while median viral load dropped below detection (Table 3.1).

**Table 3.1. Characteristics of 39 infants from the OPH cohort during initial 24 months of ART.**

| | ART initiation | | | After 24 months of ART | | |
|---|---|---|---|---|---|---|
| **Infant ID** | **Age** (Months) | **CD4** (%) | **HIV RNA** ($log_{10}$C/mL)[a] | **CD4** (%) | **HIV RNA** ($log_{10}$C/ mL)[a] | **HIV DNA** (C/$10^6$PBMCs)[b] |
| **19** | 2.5 | 33 | 6.48 | 22 | bd | 60 |
| **32** | 2.5 | 16 | 6.96 | 28 | bd | 154 |
| **22** | 2.7 | 38 | 6.50 | 35 | bd | 77 |
| **73** | 3.3 | 14 | 7.26 | 41 | bd | 234 |
| **83** | 3.3 | 26 | 5.83 | 40 | bd | 244 |
| **60** | 3.6 | 8 | 6.30 | 33 | 2.71 | 1,259 |
| **3** | 4 | 16 | 6.42 | 36 | bd | 39 |
| **7** | 4 | 18 | 7.68 | 38 | bd | 58 |
| **77** | 4.2 | 14 | 7.52 | 29 | bd | 562 |
| **29** | 4.3 | 24 | 7.19 | 37 | bd | 497 |
| **132** | 4.3 | 25 | NA | 38 | bd | 807 |
| **5** | 4.4 | 28 | 6.29 | 44 | bd | 290 |
| **76** | 4.4 | 19 | 5.98 | 37 | bd | 165 |
| **46** | 4.5 | 23 | 7.51 | 48 | 2.23 | 967 |
| **49** | 4.5 | 15 | 5.36 | 40 | bd | 168 |
| **57** | 4.5 | 17 | NA | 30 | 2.82 | 199 |
| **34** | 4.6 | 15 | 6.93 | 28 | bd | 930 |
| **47** | 4.6 | 17 | 6.94 | 31 | bd | 868 |
| **50** | 4.7 | 14 | 6.47 | 13 | bd | 251 |
| **55** | 4.8 | 9 | 6.96 | 27 | bd | 485 |
| **1** | 4.9 | 13 | NA | 38 | bd | 141 |
| **35** | 4.9 | 23 | 5.52 | 39 | bd | 418 |
| **12** | 5 | 26 | 6.72 | 36 | bd | 344 |
| **28** | 5 | 23 | 6.30 | 33 | bd | 163 |
| **4** | 5.2 | 10 | 7.14 | 24 | bd | 155 |
| **10** | 5.3 | 39 | 5.46 | 51 | bd | 533 |
| **27** | 5.4 | 21 | 7.32 | 30 | 2.57 | 494 |
| **105** | 6.9 | 22 | 6.11 | 33 | bd | 18 |
| **117** | 7 | 15 | 6.66 | 20 | bd | 68 |
| **124** | 7 | 32 | 5.45 | 33 | bd | 494 |
| **101** | 7.1 | 24 | 5.03 | 24 | bd | 25 |
| **130** | 7.3 | 22 | 6.59 | 41 | bd | 180 |
| **109** | 7.5 | 29 | 6.12 | 33 | 2.64 | 260 |
| **123** | 7.8 | 22 | NA | 38 | bd | 420 |
| **129** | 8.1 | 14 | 5.88 | 24 | bd | 37 |
| **116** | 10 | 34 | NA | 43 | bd | bd |
| **141** | 10 | 23 | NA | 34 | bd | 90 |
| **110** | 10.2 | 12 | 6.48 | 14 | bd | 1,209 |
| **128** | 11.3 | 25 | 6.22 | 34 | bd | 137 |
| **Median** | 4.8 | 22 | 6.5 | 35 | bd | 234 |

**C = Copies, NA= Not available, bd= Below detection, [a] HIV RNA assay limit of detection is 2.18$log_{10}$ copies/mL, [b] HIV DNA assay limit of detection is 5 copies/$10^6$ PBMCs.**

***Pre-ART Correlates of reservoir size***

In order to assess whether initiating ART early after HIV infection or pre-ART levels of ADCC are associated with reservoir size, we measured HIV DNA levels twenty-four months after ART initiation in the 39 infants described above. HIV DNA reservoir was detectable in thirty-eight out of thirty-nine children. One child was below the limit of detection for the assay (5 c/$10^6$ PBMCs). Median ART initiation was 4.8 months (range 2.5-11.3). Infants treated prior to 3 months of age (n=3) had a smaller HIV DNA reservoir size (Median=77copies of HIV DNA/$10^6$ PBMCs) than those treated after 3 months (n=36) of age (Median=248copies of HIV DNA/$10^6$ PBMCs); however, these median latent reservoir measurements were not statistically different (p=0.1 Wilcoxon Rank Sum Test, Figure 3.1), likely due to the limited number of infants in the early treatment group.

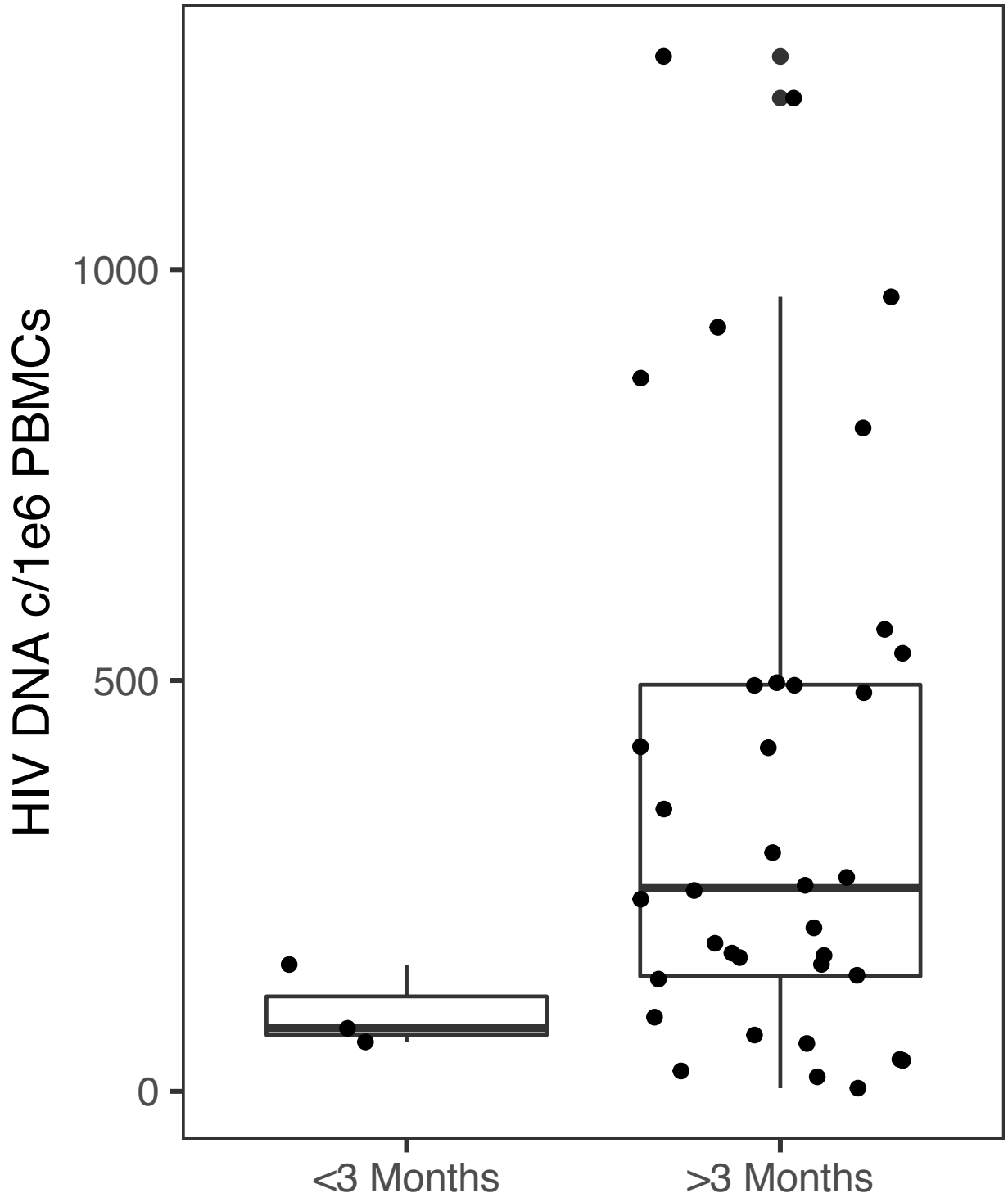


**Figure 3.1. HIV DNA reservoir of infants treated prior to three months of age compared to infants treated after three months of age.**
Infants treated within 3 months of age had lower a median level of HIV DNA (median 77 copies of HIV DNA/ $10^6$ PBMCs) than those treated between 3 and 12 months of age (248 copies of HIV DNA/$10^6$ PBMCs), p=0.1.

***Passively acquired ADCC activity in children infected between 3 and 12 months of age was low***

To assess the impact of passively acquired pre-ART HIV specific ADCC-mediating antibodies on HIV DNA reservoir size we measured HIV specific plasma ADCC activity in 25 of the 39 plasma samples just prior to ART initiation. We detected ADCC activity (mean 39.9%) in 4 of 25 infants, while ADCC activity was below the limit of detection for the assay in the remaining 21 infants (Figure 3.2A). ADCC activity was highest in infants initiating ART at 2.5 months of age, perhaps because we were detecting both passive and de novo responses, and when compared to HIV DNA at 24 months post ART initiation there was not a significant correlation between ADCC activity and HIV DNA reservoir size (Spearman's rho = 0.02, p=0.93, Figure 3.2B).

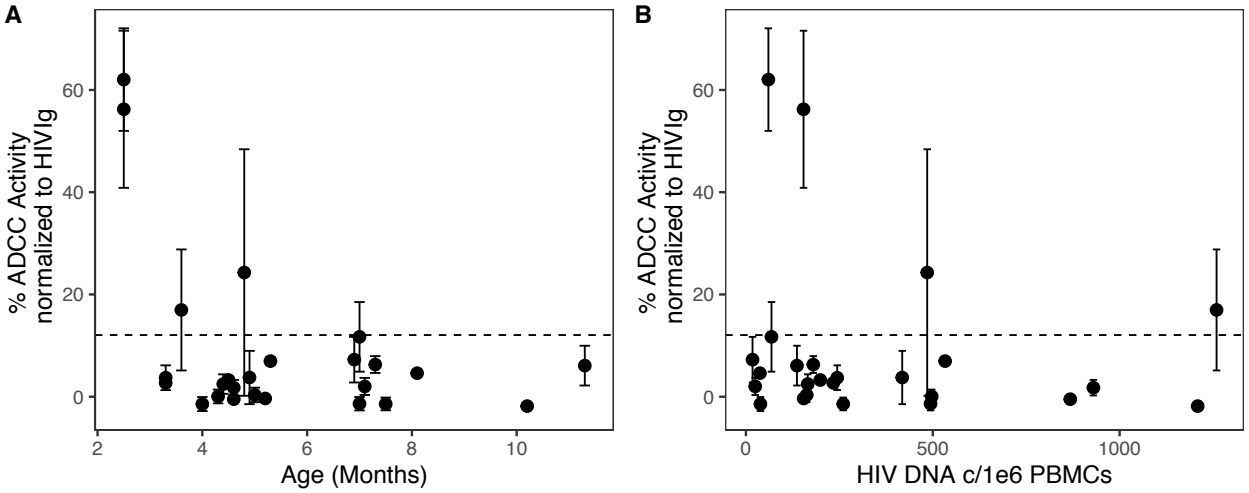


**Figure 3.2. Passively acquired HIV specific ADCC activity of 25 infants in relation to (A) age and (B) HIV DNA.**
Results are plotted as the mean ADCC activity normalized to the positive control HIVIg ±SD of 2 independent experiments in duplicate. The dashed line indicates the negative cutoff value for the assay which was defined as 3 times the standard deviation over the mean of HIV negative plasma at a 1:1000 dilution. In panel B ADCC values that fell below the negative cutoff were set to the midpoint between 0 and the negative cutoff.

***Cohort characteristics during initial 24 months of ART for children randomized to treatment***

***interruption***

For the TI portion of this study, 14 of the 39 infants described above were randomized to TI (n=7) or continued ART (n=7) after 24 months of ART (range 23-28 months). At ART initiation, median age of the 14 infants was 4.8 months (IQR 4.4-7), median CD4% was 22.5% (IQR 15-25) and median viral load was 6.5 $\log_{10}$ copies/mL (IQR 5.7-6.9) (Table 3.2). Initial ART was NNRTI-based for 10 infants and PI-based for 4 infants. Twenty-four months following ART initiation (the time of randomization) 2 children were still on NNRTI-based ART and 12 were taking PI-based ART; median CD4% had risen to 35% (IQR 32-40) while median viral load dropped below detection (Table 3.2).

**Table 3.2 Characteristics of 14 infants randomized to treatment interruption.**

| | | ART initiation | | | 24 months (24 months of ART) | | | 42 months (18 months after randomization) | | Between 24 and 42 months (Randomization to study end) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Infant ID** | **TI length** (Days) | **Age** (Months) | **CD4** (%) | **HIV RNA** ($\log_{10}$C/mL)[a] | **CD4** (%) | **HIV RNA** ($\log_{10}$C/ mL)[a] | **HIV DNA** (C/$10^6$PBMCs)[b] | **HIV DNA** (C/$10^6$PBMCs)[b] | **CD4** (%) | **Peak HIV RNA** ($\log_{10}$C/mL)[a] | **Fold change of HIV DNA** |
| **Continued Arm** | | | | | | | | | | | |
| **19** | 0 | 2.5 | 33 | 6.48 | 27 | bd | 60 | 8 | 64 | 6.37 | 0.13 |
| **73** | 0 | 3.3 | 14 | 7.26 | 41 | bd | 234 | 50 | 30 | bd | 0.21 |
| **49** | 0 | 4.5 | 15 | 5.36 | 40 | bd | 168 | 39 | 40 | bd | 0.23 |
| **124** | 0 | 7 | 32 | 5.45 | 33 | bd | 494 | 197 | 35 | bd | 0.40 |
| **78** | 0 | 4.7 | 22 | 7.25 | 40 | 2.41 | 237 | 150 | 27 | bd | 0.63 |
| **1** | 0 | 4.9 | 13 | NA | 38 | bd | 141 | 877 | 43 | 6.88 | 6.21 |
| **116** | 0 | 10 | 34 | NA | 43 | bd | bd | bd | 41 | bd | NA |
| **Median** | 0 | 4.70 | 22 | 6.48 | 40 | bd | 168 | 50 | 40 | bd | 0.32 |
| **Treatment Interruption Arm** | | | | | | | | | | | |
| **34** | 116 | 4.6 | 15 | 6.93 | 28 | bd | 930 | 240 | 28 | 6.60 | 0.26 |
| **101** | 130 | 7.1 | 24 | 5.03 | 32 | bd | 25 | 14 | 35 | 4.82 | 0.57 |
| **132** | 101 | 4.3 | 25 | NA | 38 | bd | 807 | 693 | 42 | 6.55 | 0.86 |
| **141** | 106 | 10 | 23 | NA | 34 | bd | 90 | 94 | 36 | 6.02 | 1.04 |
| **117** | 122 | 7 | 15 | 6.66 | 26 | bd | 68 | 90 | 28 | 6.38 | 1.31 |
| **28** | 106 | 5 | 23 | 6.30 | 33 | bd | 163 | 321 | 33 | 6.07 | 1.97 |
| **3** | 101 | 4 | 16 | 6.42 | 36 | bd | 39 | 185 | 34 | 6.26 | 4.74 |
| **Median** | 106 | 5 | 23 | 6.42 | 33 | bd | 90 | 185 | 34 | 6.26 | 1.04 |

C = Copies, NA= Not available, bd= Below detection, [a] HIV RNA assay limit of detection is 2.18$\log_{10}$ copies/mL, [b] HIV DNA assay limit of detection is 5 copies/$10^6$ PBMCs.

***Treatment interruption***

Fourteen of the 39 infants were randomized to TI or continued therapy. At the first post-randomization visit (~3 months after TI), all 7 children in the TI arm exhibited viral rebound (Figure 3.3A) and met CD4-based criteria to restart ART, resulting in a median 106 days (IQR 104-119) off ART. Of the 7 children in the continued arm, 5 had continuous viral suppression while 2 had high HIV RNA levels at a single time-point: infant #19 at 3 months and #1 at 9 months post-randomization (Figure 3.3A). Three months after randomization, median viral load was 6.3 $\log_{10}$ copies/mL and below detection (<2.18 $\log_{10}$ copies/mL), and median CD4 was 21% and 42%, in the TI and continued arms, respectively. Eighteen months after randomization, all 14 children were on PI-based ART with viral loads below detection. The median CD4% was 34% (IQR 30.5-35.5) in the TI group and 40% (IQR 32.5- 42) in the continued group.

***HIV DNA reservoir dynamics following short treatment interruption***

To identify whether short treatment interruption causes an increase in the HIV DNA reservoir we compared the change in HIV DNA reservoir size, before randomization and 18 months after, between children randomized to TI (n=7) or continued ART(n=7). After 24 months of initial ART, children randomized to continued ART had a median 168 HIV DNA copies/$10^6$ PBMCs (IQR: 101-235) in the reservoir and children in the TI arm had median 90 HIV DNA copies/$10^6$ PBMCs (IQR: 54-485) (p=0.9). Eighteen months later, after children in the TI arm had resumed ART for >15 months, HIV DNA levels were median 50 HIV DNA copies/$10^6$ PBMCs (IQR: 24-174) and 185 HIV DNA copies/$10^6$ PBMCs (IQR: 92-280) in the continued and TI arms, respectively (p=0.53). During the 18 months after randomization, 1 of 7 children in the continued

arm had increased HIV DNA, 5 of 7 had decreased HIV DNA (range 0.13-0.63), and 1 of 7 children had HIV DNA levels below detection at both time points (Figure 3.3B, Table 3.2). In the TI arm, 3 of 7 children had increases in HIV DNA (range 1.3-4.7), while 4 of 7 had decreased or unchanged HIV DNA levels (range 0.26-1.04). Median HIV DNA fold change was 0.32 (IQR: 0.22-0.58) in children randomized to continued ART and 1.04 (IQR: 0.72-1.64) in children randomized to TI (p=0.14) (Figure 3.3C). Similar results were observed when we excluded the 2 children in the continued arm with post-randomization viremia: median HIV DNA fold change was 0.32 (IQR: 0.23-0.46) versus 1.04 (IQR: 0.71-1.64) in continued and TI arms, respectively (p=0.04). In children with viremia during the 18 months after randomization, change in HIV DNA did not correlate with peak viremia (Spearman's rho = 0.12, p=0.77).

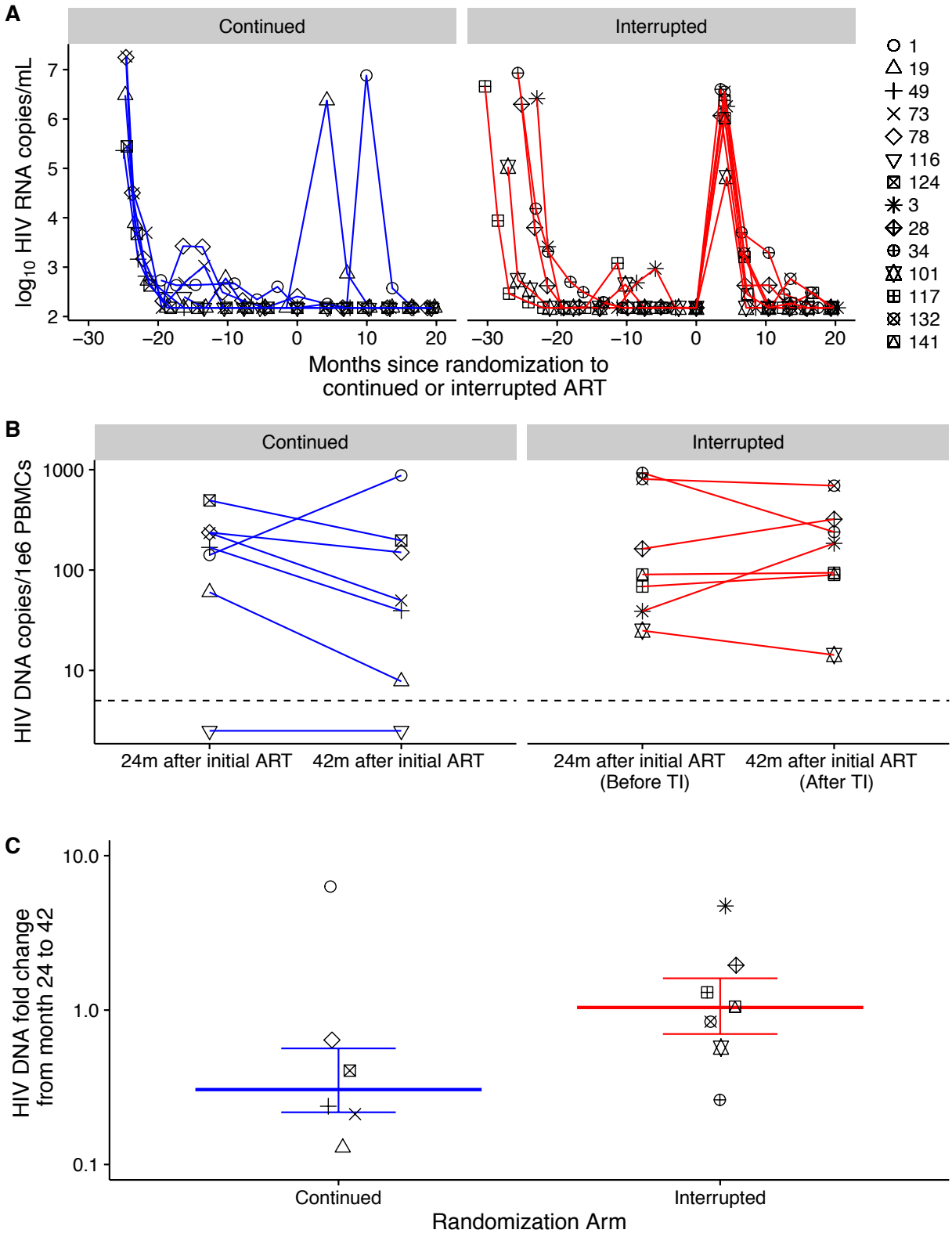


**Figure 3.3. HIV RNA and DNA in infants randomized to treatment interruption.**
(A) Plasma HIV RNA copies/mL over time in infants randomized to continued ART (left) or interrupted ART (right). Limit of detection was 2.18 log10 copies HIV RNA/mL. (B) Comparison

of total HIV DNA copies/$10^6$ PBMCs before (24-month visit) and after (42-month visit) randomization to treatment interruption in infants in the continued (left) or interrupted arm (right). Limit of detection was 5 copies of HIV DNA/$10^6$ PBMCs. (C) HIV DNA fold change following randomization to treatment interruption (comparing HIV DNA at the 24 and 42 month visits) in infants randomized to continued (left) or interrupted ART (right).

In a limited number of children with longer follow-up (2 in the TI arm and 5 in the continued arm), a virally suppressed sample was available from 51 months after randomization (75 months after initial ART). The median HIV DNA declines from 0 to 51 months following randomization were –1.96 HIV DNA copies/$10^6$ PBMCs/month in the continued arm and –0.37 in the interrupted arm. The median HIV DNA fold changes were 0.44 (IQR, 0.44–0.75) and 0.92 (IQR, 0.84–1.00) in children randomized to continued ART and TI, respectively (Figure 3.4).

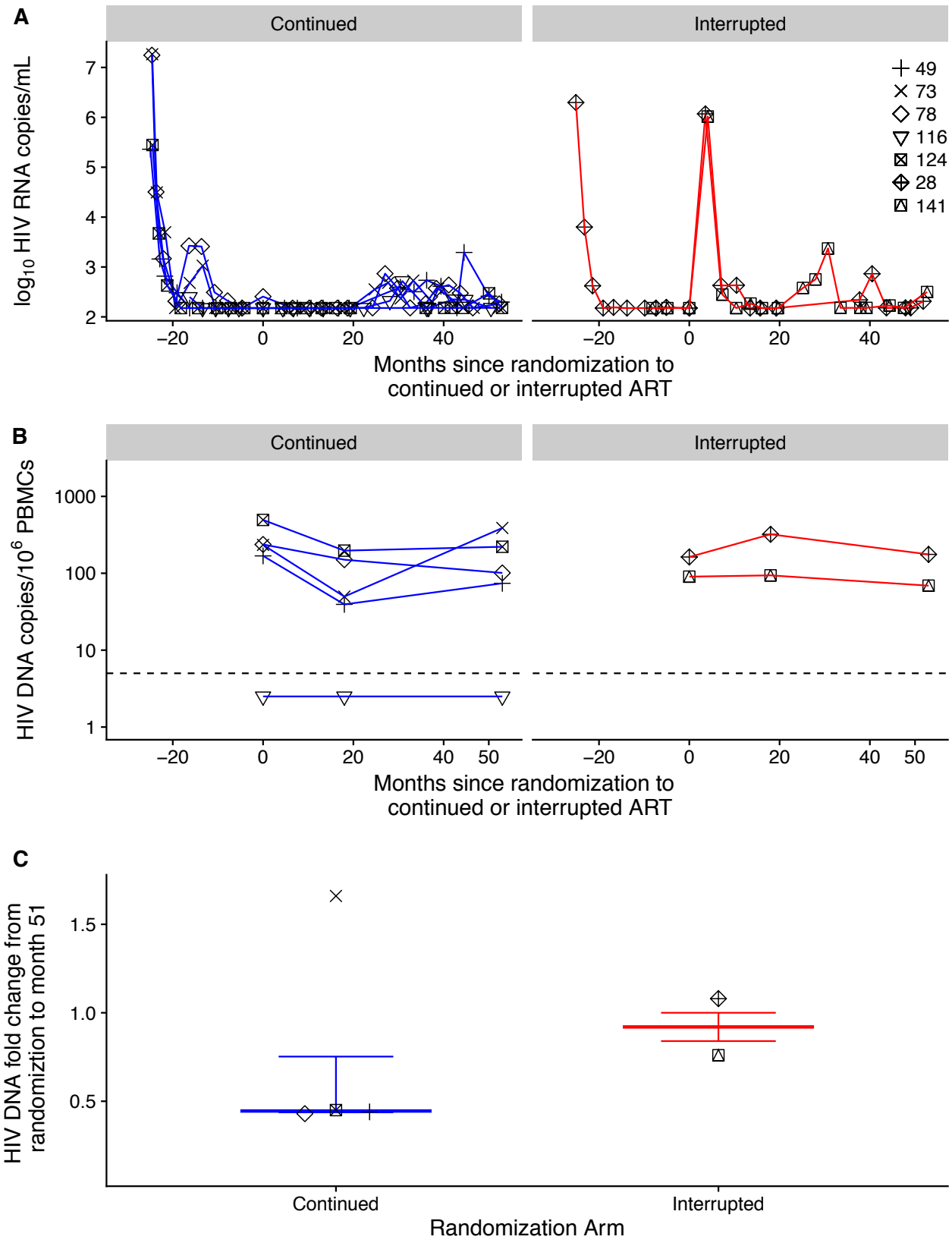


**Figure 3.4. Extended HIV RNA and DNA in infants randomized to treatment interruption.**

(A) Plasma HIV RNA copies/mL over time in infants randomized to continued ART (left) or treatment interruption (right). Limit of detection was 2.18 log10 copies HIV RNA/mL. (B) Comparison of total HIV DNA copies/$10^6$ PBMCs before (24 month after initial ART) and after randomization to treatment interruption (18 and 51 months later) in children in the continued (left) or interrupted arm (right). Limit of detection was 5 copies of HIV DNA/$10^6$ PBMCs. (C) HIV DNA fold change following randomization to treatment interruption (comparing HIV DNA at randomization and 51 months later) in infants randomized to continued (left) or interrupted ART (right).

***ADCC activity during TI is not associated with change in HIV DNA reservoir size***

To test whether *de novo* ADCC activity during treatment interruption influenced HIV DNA reservoir size we compared HIV specific ADCC activity in the TI group to the fold change in HIV DNA between randomization and 18 months after. We detected high levels of HIV specific ADCC activity (mean 80%) in all 7 children randomized to TI compared to no HIV specific ADCC activity in children randomized to continued ART. Change in HIV DNA did not correlate with *de novo* HIV specific ADCC activity in children randomized to TI (Spearman's rho = 0.21, p = 0.66) (Figure 3.5).

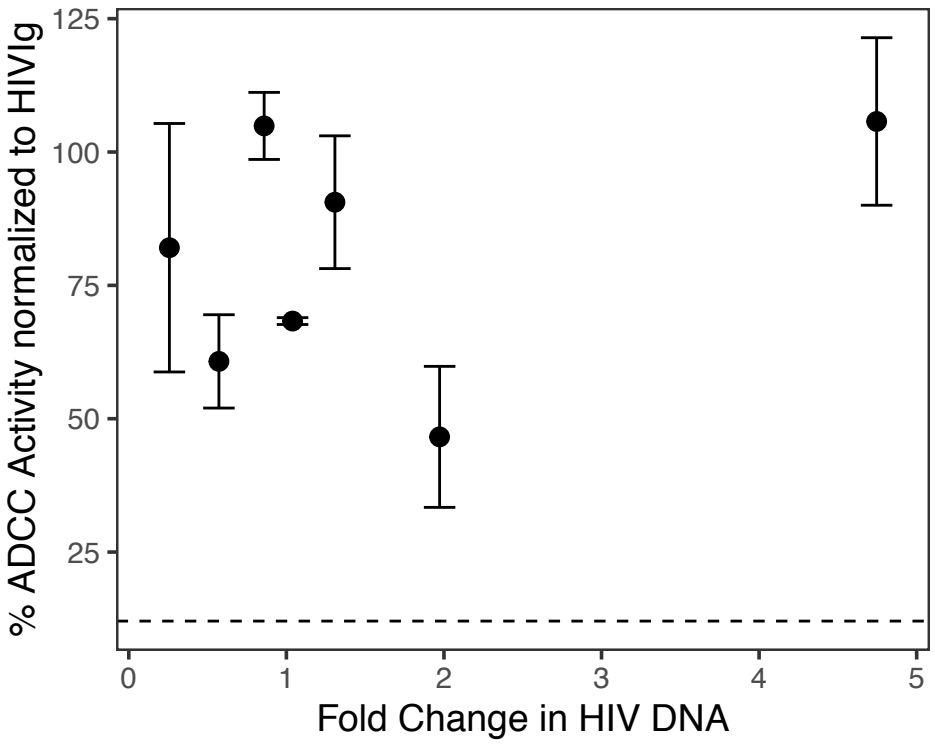


**Figure 3.5. *de novo* HIV specific ADCC activity compared to fold change in HIV DNA during treatment interruption.**
Results are plotted as the mean ADCC activity normalized to the positive control HIVIg ±SD of 2 independent experiments in duplicate. The dashed line indicates the negative cutoff value for the assay.

## 3.4 Discussion

HIV latent reservoir size has been shown to be limited by ART initiated very early in infection in both infants, and adults [54, 57, 115-121], however, early ART alone is not sufficient to insure small reservoir size [122] and the role of host immune responses, such as HIV specific ADCC mediating antibodies, is less well known [72]. To identify pre-ART correlates of HIV DNA reservoir size, we measured the HIV DNA reservoir 24 months after ART initiation as well as pre-ART ADCC levels in perinatally infected infants that initiated ART between 2.5-11.3 months of age. We observed that children treated prior to three months of age had lower levels of HIV DNA

than those treated after three months of age. Although our association was not significant, due to the limited number of infants treated prior to 3 months of age in our cohort, our data supports the numerous studies that suggest early ART limits the HIV latent [54, 57, 115-121]. The most famous example of early ART limiting reservoir size, is the case of the "Mississippi baby"; who was placed on ART within 30 hours of birth, achieved ART free remission following 18 months of treatment, and had undetectable levels of HIV DNA following TI [69] suggesting that a functional cure might be possible with immediate ART in perinatally infected infants. In our study, while children treated earlier had lower levels of HIV DNA, one child treated at 10 months of age had undetectable HIV DNA in 2.5e5 PBMCs, suggesting that there are other potential mechanisms that limit reservoir size.

We compared passively acquired ADCC activity in perinatally infected infants at the time of ART initiation to HIV DNA reservoir size and observed that 21 out of 25 infants had ADCC activity below the limit of detection for our assay. The two infants with the highest levels of ADCC activity were also the 2 infants with the youngest age at ART initiation: 2.5 months of age. A previous study from our group observed that in young HIV-exposed infants, HIV specific ADCC activity declined to undetectable levels 2 to 4 months following birth [112] as passively transferred maternal antibodies waned prior to the HIV-infected infant's de novo generation of HIV specific ADCC mediating antibodies at 6-12 months of age. Since most of our samples were obtained between 4-8 months of age, it is likely that our samples are from after the time at which passively acquired maternal antibodies have decayed, and prior to de novo antibody generation. Thus, we are not able to conclude whether levels of pre-ART HIV-specific ADCC mediating antibodies correlate with HIV DNA reservoir size, as this relationship would be better analyzed in a cohort with plasma samples from just following birth.

Individuals who achieve limited reservoir size can potentially benefit the most from HIV cure studies seeking to eradicate latently infected cells. Barring the development of an accurate and sensitive assay to identify the absence of latently infected cells, treatment interruption will be necessary to assess HIV cure study efficacy. However, the benefits of early treatment may be eliminated by reservoir expansion during treatment interruption due to infection of new cells and subsequent transition into latency. Therefore, we measured HIV DNA in the reservoir in children that started ART during the first year of life and were randomized 2 years later to continue or interrupt ART. We compared HIV DNA levels at randomization and again 18 months later, after all children had resumed ART for >15 months and achieved viral suppression. HIV DNA decayed in children with continued viral suppression, while the median HIV DNA fold change after TI was 1.04, suggesting TI lessens the decay that occurs on continued ART. Indeed, for 2 children in the TI arm with ~4 years of follow-up, HIV DNA reservoir size remained relatively unchanged over time (Figure 3.4). The fact that most children in the TI arm had similar HIV DNA levels before and after TI, suggests that reservoir re-seeding that occurred during TI was followed by decay after ART resumption.

We also measured HIV specific ADCC activity three months after TI to identify whether reservoir re-seeding was correlated with ADCC mediated killing of reservoir cells during TI. We found no correlation with HIV specific ADCC activity and change in HIV DNA reservoir size, suggesting that de novo HIV specific ADCC activity during TI did not limit reservoir seeding. However, we were only able to assess ADCC during TI in a small number (n=7) of infants and studies with larger numbers of infants, or infants treated within hours of birth may have different outcomes.

The fact that most children in the TI arm had similar HIV DNA levels before and after TI, suggests that reservoir re-seeding that occurred during TI was followed by decay after ART resumption. Mechanisms of reservoir seeding include new infection of activated CD4+ T cells that become quiescent and clonal proliferation of cells containing provirus [37, 38]. Our study could not distinguish between these mechanisms as we did not characterize the reservoir composition or replication competence due to limited sample volume and cell viability. In addition, our cohort did not include infants treated with very early ART (within hours of birth) which may have different viral reservoir decay dynamics.

Our study did not quantify HIV DNA in tissue reservoirs and was limited by small sample size with only two evaluated time points. However, our data adds substantially to the two previous studies on changes in HIV DNA following TI in children [55, 56], and supports results from adult cohorts [78, 79]. Analysis of 15 adults in the SPARTAC trial with transient TI showed HIV DNA returned to pre-TI levels after ≥6 months of ART [79]. Another TI study of 10 adults with very low HIV DNA levels in the reservoir prior to a median TI of 4 weeks observed similar results, with HIV DNA levels returning to pre-TI values after treatment resumption [78]. These studies suggest increases in HIV DNA can be minimized or reversed by rapid treatment resumption, however, studies with longer follow-up are needed.

Our findings add a new perspective that complements prior TI studies in perinatally infected infants, which focused on longer TI. The largest pediatric TI reservoir study to date included 17 infants treated earlier (<12 weeks at ART initiation) and with longer TI (median 11 months) than in our study, and observed increased HIV DNA in the reservoir 26 months after treatment resumption [55]. Another study included only 3 infants with TI, and showed 52.4, 1.8 and 12.2-fold increases in HIV DNA following TI of 0.75, 6.8 and 71 months, respectively [56].

Here we show short interruption of ~3 months does not appear to have sustained impact on HIV DNA levels in the reservoir in infants. This data suggests re-seeding of the reservoir in pediatric HIV may be minimized with frequent viral load monitoring during short analytical TI.

# Chapter 4. Timing of HIV DNA reservoir seeding in a cohort of superinfected Kenyan women

## 4.1 Introduction

A major obstacle precluding an HIV cure is the establishment of a latent reservoir of HIV infected cells that persist despite suppressive antiretroviral therapy (ART) [123]. Most ART intensification and proviral sequencing studies suggest that there is little to no new infection of host cells while on suppressive ART [124-126]. Persistence is instead driven by infection of long-lived resting CD4+ T cells and clonal proliferation [36-38], leading to a stable reservoir with an estimated half-life of ~44 months [83]; necessitating lifelong therapy to prevent viral recrudescence. Understanding when and how the reservoir is seeded is essential to inform HIV cure studies.

Multiple studies have shown that the reservoir is established early in infection, including a study of SIV infection in macaques that showed the reservoir is formed prior to detection of viremia in blood, but may be limited in size and location by early treatment [53, 127]. Studies in humans have also found that treatment early during acute infection reduced the latent reservoir size compared to later treatment [109, 128], and may contribute to an increased incidence of post-treatment controllers [54].

While many studies have shown that early treatment limits reservoir size, less is known about HIV latent reservoir seeding dynamics. Some studies have suggested that the reservoir is seeded predominantly early, due to the presence of early viral variants in provirus outgrowth or sequencing of proviral DNA from PBMCs [59, 129], while others suggest the reservoir is continually seeded throughout infection based on introduction of drug resistance into the reservoir during sub-optimal treatment [58], or modeling of RNA and DNA sequences throughout infection

and subsequent treatment [130]. However, recent studies that compared latent reservoir sequences to longitudinal HIV RNA sequences have observed that the majority of latent reservoir sequences are most closely related to HIV RNA sequences circulating just prior to the initiation of ART [60, 61]. These studies rely mainly on phylogenetic inference from the evolution of a single HIV variant to establish the temporal relationship and seeding dynamics of the latent reservoir.

HIV superinfection represents a unique setting to evaluate the timing of reservoir seeding because the individual is infected initially by one HIV strain and then at a later time with a different and genetically distinct viral strain [131]. Thus, the relative abundance and evolution of the two viral strains that established infection at different times in the reservoir can be assessed to inform phylogenetic inference and help clarify HIV latent reservoir seeding dynamics.

In this study, we compared longitudinal plasma HIV RNA sequences to on ART HIV DNA sequences in a cohort of Kenyan HIV superinfected women with known infection and superinfection timing [132-134]. We detected viruses from both the initial and superinfection lineages in the HIV DNA reservoir, confirming that reservoir seeding occurs throughout HIV infection. In addition, we found that the majority of HIV DNA sequences were most closely related to RNA sequences found in plasma at time points close to ART initiation.

## 4.2 Methods

***Study population***

Cases of HIV superinfection were identified within a cohort of women at high risk of HIV infection in Mombasa, Kenya, and timing of both initial infection and superinfection were estimated as described previously [132-134]. Cases of HIV superinfection were included in this sub-study based upon the following criteria: 1) follow-up includes time before and after ART

initiation, 2) longitudinal plasma samples available between initial infection and ART initiation with a mean of 2 years between sample time points, 3) PBMC sample available following ≥6 months of ART mediated viral suppression (plasma HIV RNA $<10^3$ copies/mL).

***HIV genome amplification and sequencing***

HIV virions were isolated using the μMACS VitalVirus HIV Isolation Kit (Miltenyi Biotec) from 10-400μl of plasma to enable isolation of an estimated ~4,000 viral particles based on an Aptima HIV-1 viral load assay (Hologic). RT-PCR was performed in duplicate to amplify ~500bp regions of gag and *env*, as previously described [132]. HIV DNA was isolated from PBMCs by Qiagen DNA Midi Kit. Droplet digital PCR was performed on an aliquot of the purified DNA to determine HIV DNA genome copy number in each sample, and an estimated 125 HIV DNA genomes were input into quadruplicate PCR reactions to amplify *gag* and *env* regions (as above) from ~500 HIV DNA genomes in total. Nextera adaptors were added by 10 cycles of PCR using Kappa HiFi Polymerase. PCR products were cleaned by AMPure XP purification beads with 1:1 sample to bead ratio, quantified by Qubit dsDNA HS kit and equal amounts pooled into 20μl for barcode addition. Illumina barcodes were then added by PCR ligation to uniquely identify each sample, cleaned by AMPure XP purification beads, and pooled. The pooled library was gel isolated using a Zymoclean kit and sequenced on an Illumina Miseq using 2x300 bp paired-end reads.

***Bioinformatic pipeline***

Demultiplexing, denoising, and amplicon sequence variant clustering was performed using DADA2 [135], identical sequences were collapsed and read counts of unique variants were

determined. Common lab HIV sequences and cross-contamination were removed from the analysis by BLAST against a local database containing patient specific sequences from previous studies [132-134]. Sequences with an abundance of less than 0.5% were also removed. Consensus sequences of the initial and superinfecting lineage variants were made from sequencing data from previous studies [132-134] and used in the RAPR tool on the LANL website to enable classification of sequences from this study as initial, superinfecting, or recombinant lineage variants [136]. The initial, superinfecting, and recombinant lineage groups identified by RAPR were used to form lineage-specific consensus sequences via the Biopython (https://biopython.org/) Python package. Mutations were measured against the representative RAPR-identified lineage consensus sequence to identify hypermutant variants in the context of a given lineage. Premature stop codons were identified by aligning sampled sequences to the HXB2 (https://www.hiv.lanl.gov/content/sequence/HIV/REVIEWS/HXB2.html) HIV reference sequence to determine reading frames. Variants identified as hypermutants, as well as those containing premature stop codons or deletions of more than 50 base pairs, were filtered out of the sequencing data to control for the noise they contributed to the phylogenetic methods. Proportions of each variant were determined based on read count. Nucleotide diversity (pi), as measured by the DendroPy Python package, was compared between sampled DNA and RNA sequences to verify that sampling accounted for a comparable degree of the true variance in each virus population.

***Phylogenetic and pairwise distance analysis***

Phylogenetic trees were constructed using the maximum likelihood method with the R package phangorn[137]. Trees were then annotated using the R package ggtree[138]. Pairwise

distances between sequences were computed using the R package, Ape, and DNA sequences were matched to the RNA sequence with the smallest pairwise distance. Primary analysis was done including hypermutated sequences, secondary analysis excluding hypermutants showed similar results.

## 4.3 Results

***Longitudinal sampling of HIV superinfected women before and after ART initiation***

We previously identified cases of HIV superinfection in a prospective cohort of high-risk women in Mombasa, Kenya and determined the dates of both initial infection and superinfection [132-134]. Six of these cases, which were ART-naïve for >5 years (range 5.4-19.8), were subsequently treated with ART and achieved viral suppression for >6 months. Among these cases, time between initial infection and superinfection ranged from 0.2-5.2 years (Figure 4.1). Plasma samples from 0-2 months after the estimated date of initial infection, plus longitudinal plasma samples collected on average every 2 years (range 0.4-5.2 years) until ART initiation, as well as a PBMC sample collected after >0.9 years (range 0.9-4.8 years) of suppressive ART were available for next-generation sequencing (NGS) of *gag* and *env* (Fig 4.1), which allowed us to sample two regions of the genome because in some cases, superinfection was detected in only *gag* or *env*.

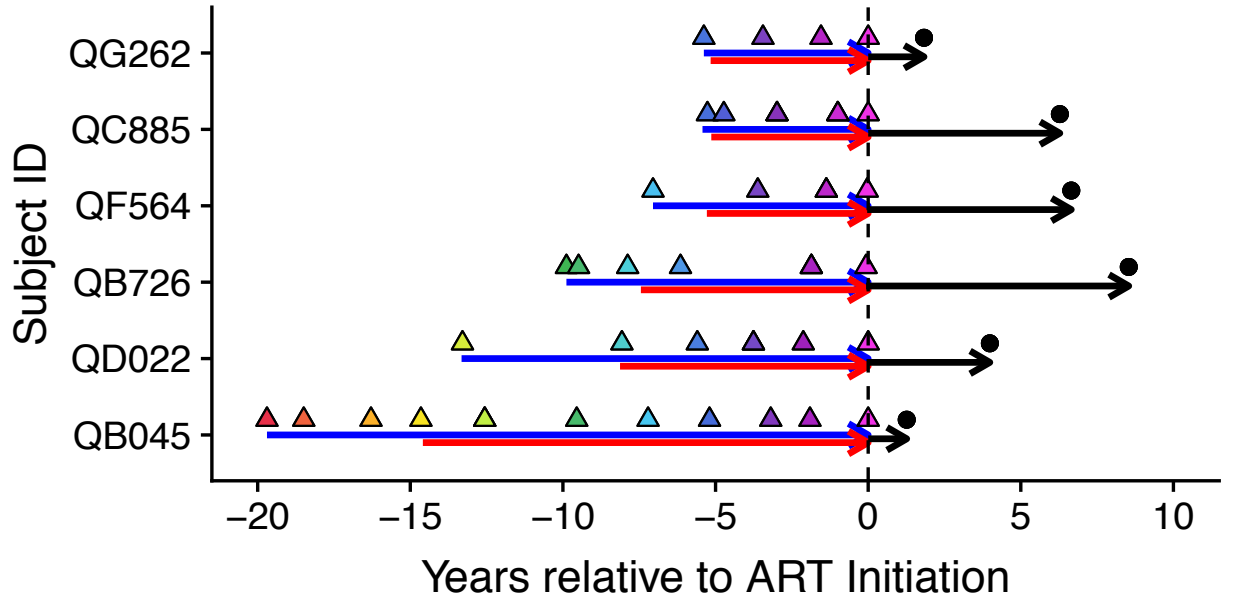


**Figure 4.1. HIV RNA and HIV DNA sample time points for the six superinfection cases in relation to ART initiation.**
Subjects were followed after initial HIV RNA detection, with horizontal lines indicating length of: initial infection (Blue) and superinfection (Red) until ART initiation; and ART (Black) initiation until the HIV DNA sample time point. Triangles indicate HIV RNA sample time points and the circle is indicative of the HIV DNA sample time point. Colors are shaded relative to ART initiation.

***Dynamics of initial and superinfecting HIV RNA lineages prior to ART initiation***

HIV RNA sequences in both *gag* and *env* were classified as lineages of the initial, superinfecting or recombinant variants. The relative proportion of each lineage varied over time following superinfection (Figure 4.2A). In *gag,* the initial and superinfecting variants co-circulated over time and formed within-region recombinants that became the dominant strain in 3 cases (QG262, QD022, and QB045), while in the other 3 cases either the superinfecting variant (QF564, QB726), or the initial variant (QC885) was dominant following superinfection (Figure 4.2A). In *env,* there were 3 cases in which the initial and superinfecting variants co-circulated over time (QG262, QB726, QB045), and in 2 of those cases a recombinant virus eventually became dominant (QG262, QB726). In the other 3 cases, the initial variant remained dominant (QC885, QF564,

QD022; Figure 4.3A) and represented more than 93% of plasma RNA sequences at all time points. The patterns in *env* did not always reflect the patterns in *gag*, suggesting between-region recombination occurred in some cases.

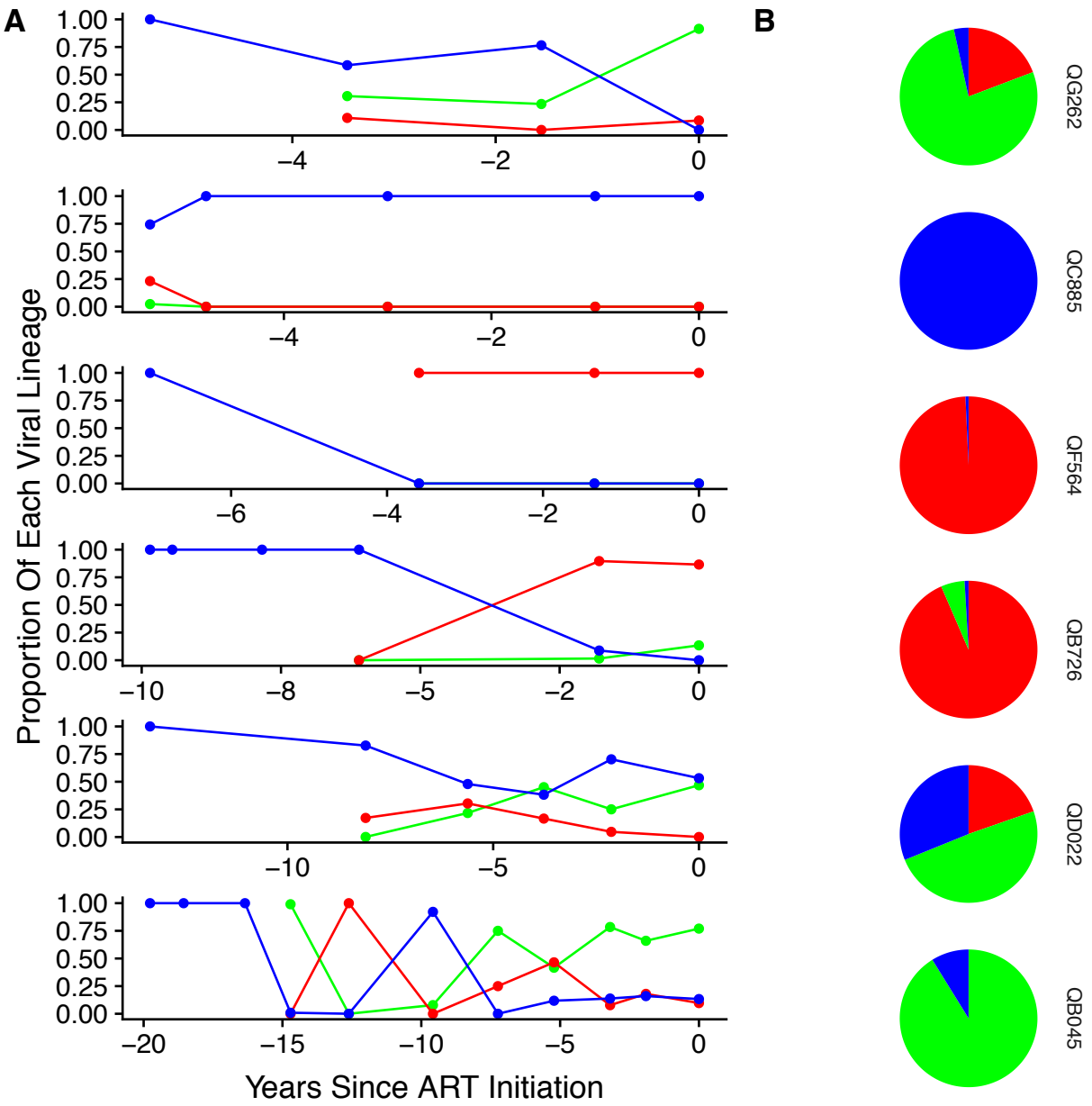


**Figure 4.2. Proportion of *gag* sequences from initial and superinfecting virus lineages prior to and during suppressive ART.**
Proportions of viral variants in (A) HIV RNA in plasma throughout infection and (B) HIV DNA present in the blood following >6 months ART initiation for the *gag* region. Blue denotes the

initial viral variant, red denotes the superinfecting variant, and green denotes within region recombinants.

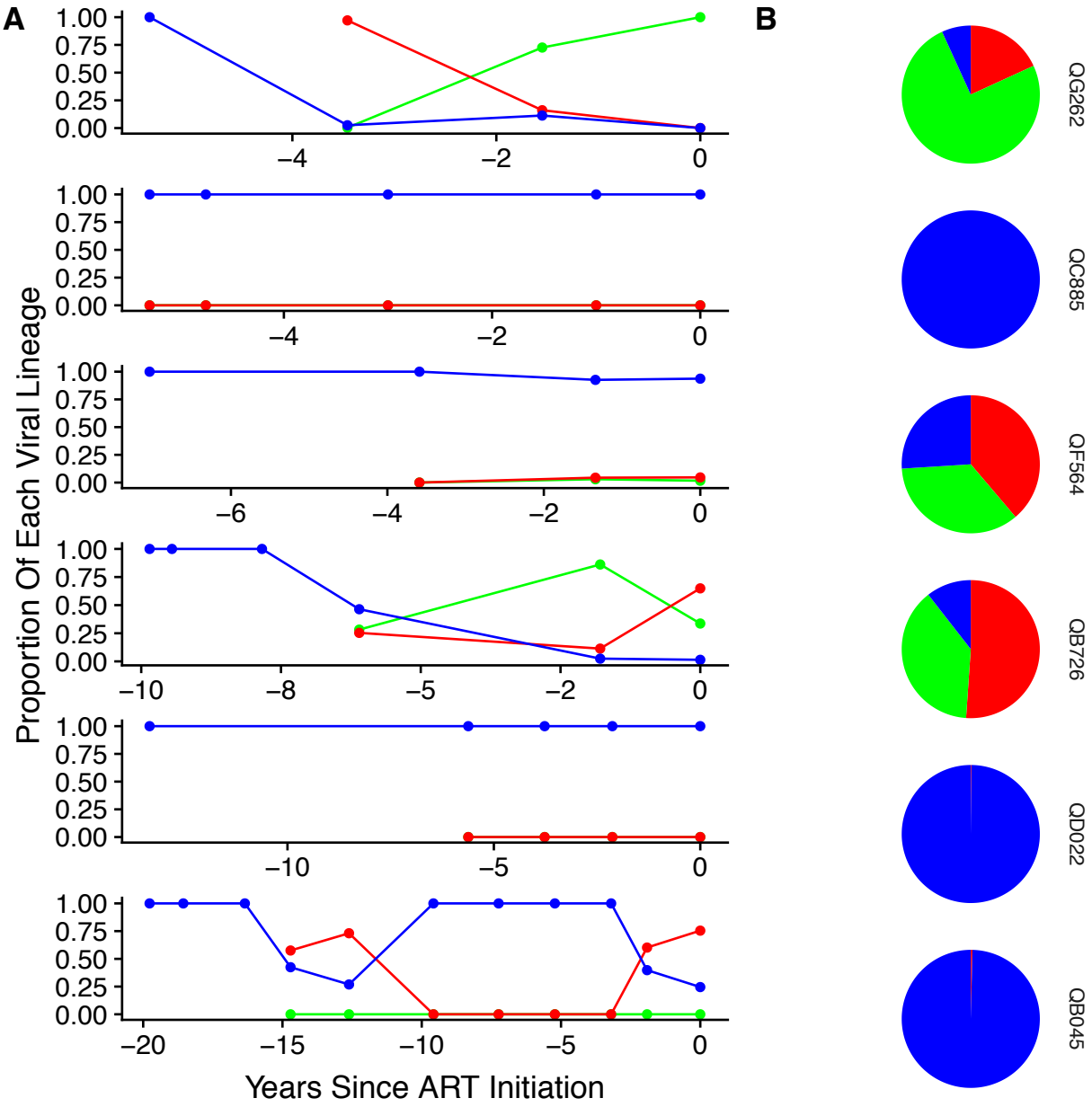


**Figure 4.3. Proportion of *env* sequences from initial and superinfecting virus lineages prior to and during suppressive ART.**
Proportions of viral variants in (A) HIV RNA in plasma throughout infection and (B) HIV DNA present in the blood following >6 months ART initiation for the *env* region. Blue denotes the

initial viral variant, red denotes the superinfecting variant, and green denotes within region recombinants.

***Initial and superinfecting variants in the HIV DNA reservoir during suppressive ART***

HIV DNA sequences in *env* and *gag* from PBMCs isolated following >6 months of viral suppression were also classified as lineages of the initial, SI or recombinant variants. In 5 of 6 cases, HIV DNA reservoir *gag* and/or *env* sequences included lineage variants from both the initial and SI virus (and/or within-region recombinants that contained both initial and SI sequences). In 1 case, QC885 in which initial lineages remained dominant in HIV RNA after superinfection, only initial virus lineages were present in both *gag* and *env* in the HIV DNA reservoir (Figure 4.2B, Figure 4.3B). In all cases, the proportion of initial, SI and recombinant variants appear similar to the proportion of each variant present at the time prior to ART initiation.

***Majority of HIV DNA reservoir sequences phylogenetically cluster with HIV RNA circulating near ART initiation***

In each case, we sampled between 50 and 349 unique variants in the RNA across all time points, and 28 and 155 unique variants in the DNA at a single time point. HIV DNA reservoir sequences phylogenetically clustered with HIV RNA in all cases in *gag* (Figure 4.4), with some DNA sequences an exact match to the RNA sequences. Similar analysis of *env* also show HIV DNA and RNA sequences are phylogenetically related (Figure 4.5). In one case, QD022, the SI variants detected in *env* DNA did not cluster with any HIV RNA sequences because the SI variant was not detected in the *env* region in RNA. In all cases, the diversity of HIV DNA sequences reflected the phylogenetic diversity of HIV RNA sequences over time (Figure 4.4, Figure 4.5),

despite the lower estimate of input HIV DNA, ~500 copies, compared to HIV RNA input, ~4000 copies, for each sample.

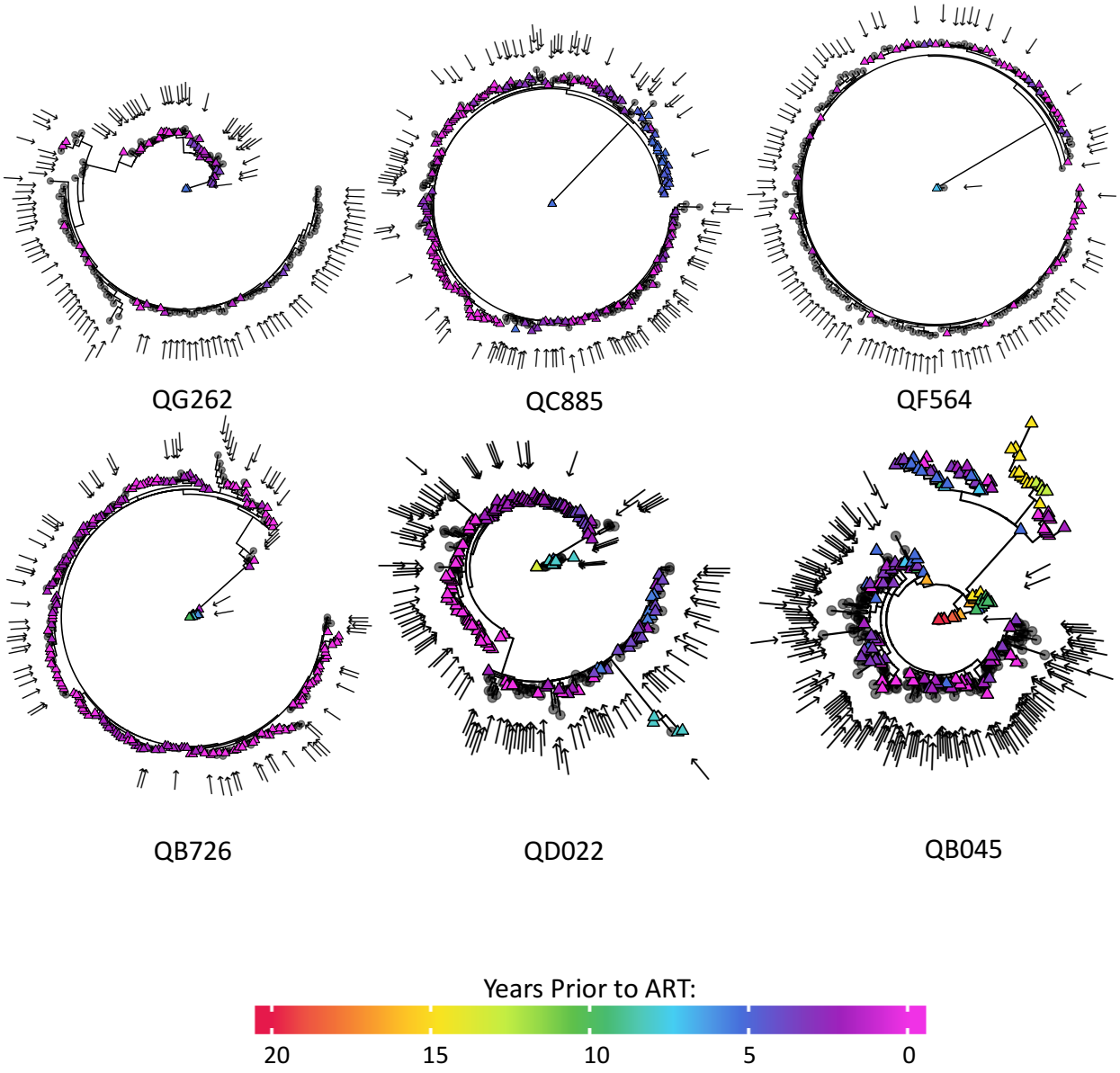


**Figure 4.4. Maximum likelihood phylogenetic trees of HIV *gag* sequences for all 6 subjects.** Sequences from pre-ART plasma HIV RNA (colored triangles) and from HIV DNA sequences (black arrows pointing at grey circles) from PBMCs collected during suppressive ART. The HIV RNA symbol colors are indicative of the time point relative to ART initiation.

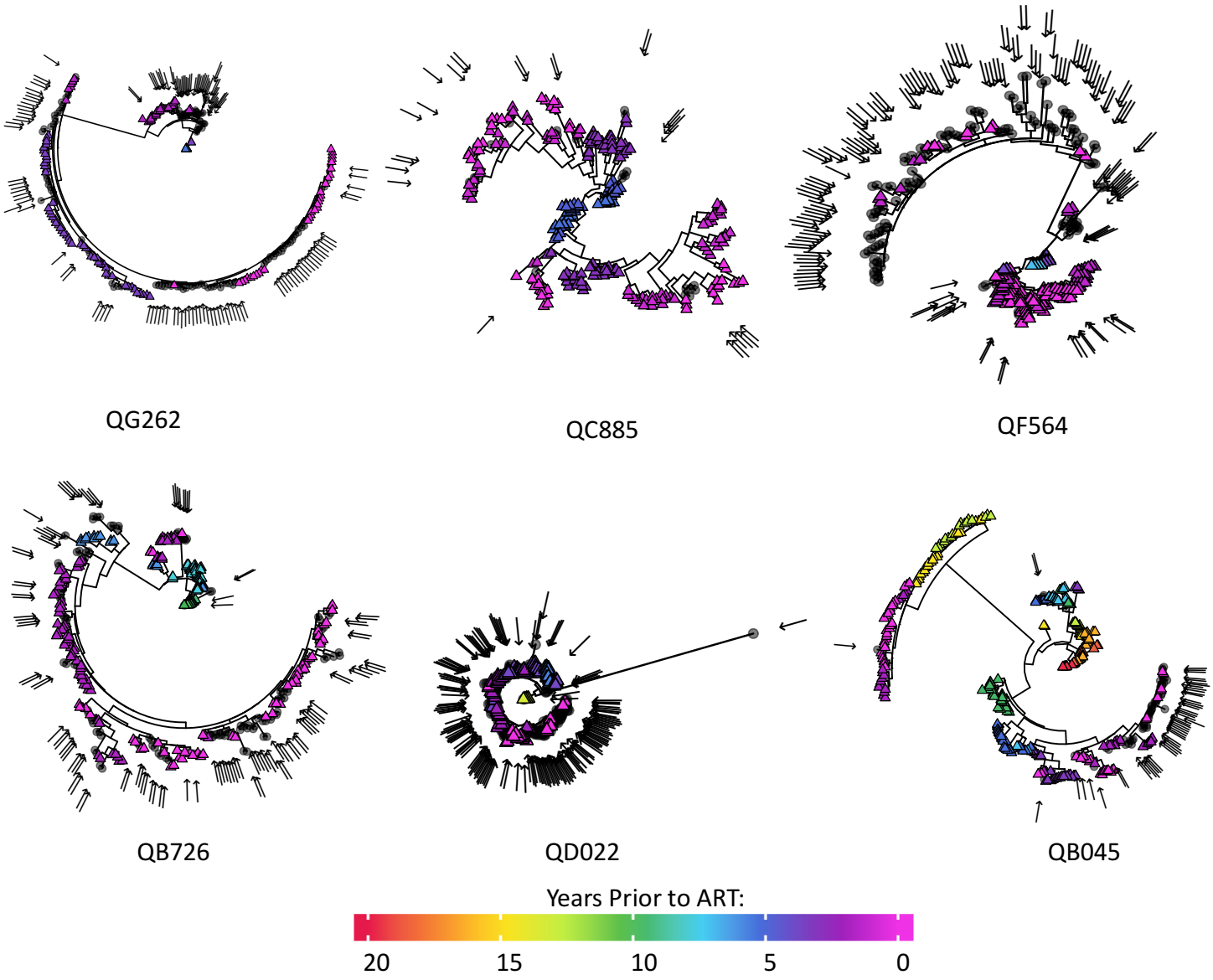


**Figure 4.5. Maximum likelihood phylogenetic trees of HIV *env* sequences for all 6 subjects.** Sequences from pre-ART plasma HIV RNA (colored triangles) and from HIV DNA sequences (black arrows pointing at grey circles) from PBMCs collected during suppressive ART. The HIV RNA symbol colors are indicative of the time point relative to ART initiation.

Pairwise distance analysis between HIV DNA and RNA sequences was used to estimate seeding time of each HIV DNA reservoir sequence. The HIV DNA sequences associated with each time point include initial, SI and/or recombinant variants that reflect the composition of the HIV RNA variants from that time point (*gag* see Figure 4.2 and 4.6A, *env* see Figure 4.3 and 4.7A). In all 6 cases, the majority of HIV DNA reservoir sequences were most closely related to HIV RNA sequences from the time of ART initiation and/or the time point prior (range 1.0-1.9 years) to ART start (*gag* see Figure 4.6A, *env* see Figure 4.7A). Combining the data from all 6 cases, we observed that greater than 55% of HIV DNA reservoir sequences in the *gag* region and 66% in *env* were most closely related to HIV RNA present at the time of ART initiation and the prior time point (*gag* see Figure 4.6B, *env* see figure 4.7B).

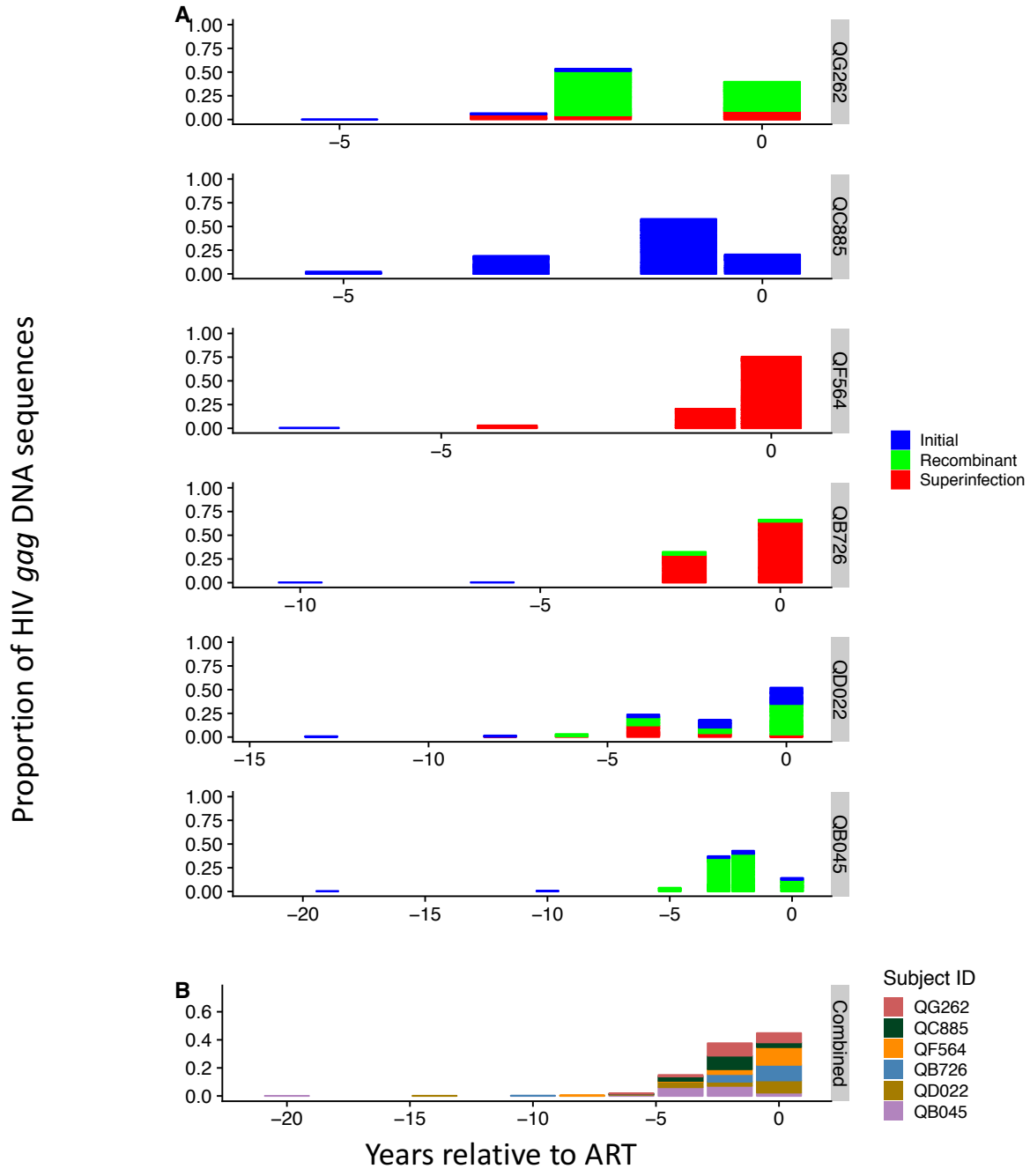


**Figure 4.6. Estimated time of HIV DNA *gag* seeding in relation to ART initiation.** Proportions of HIV DNA reservoir *gag* sequences plotted against their likely time of seeding as determined by smallest pairwise distance to longitudinal HIV RNA sequences for each Subject in (A) with combined data rounded to the nearest 2-year interval in (B). In panel A blue denotes the initial viral variant, red denotes the superinfecting variant, and green denotes within region recombinants.

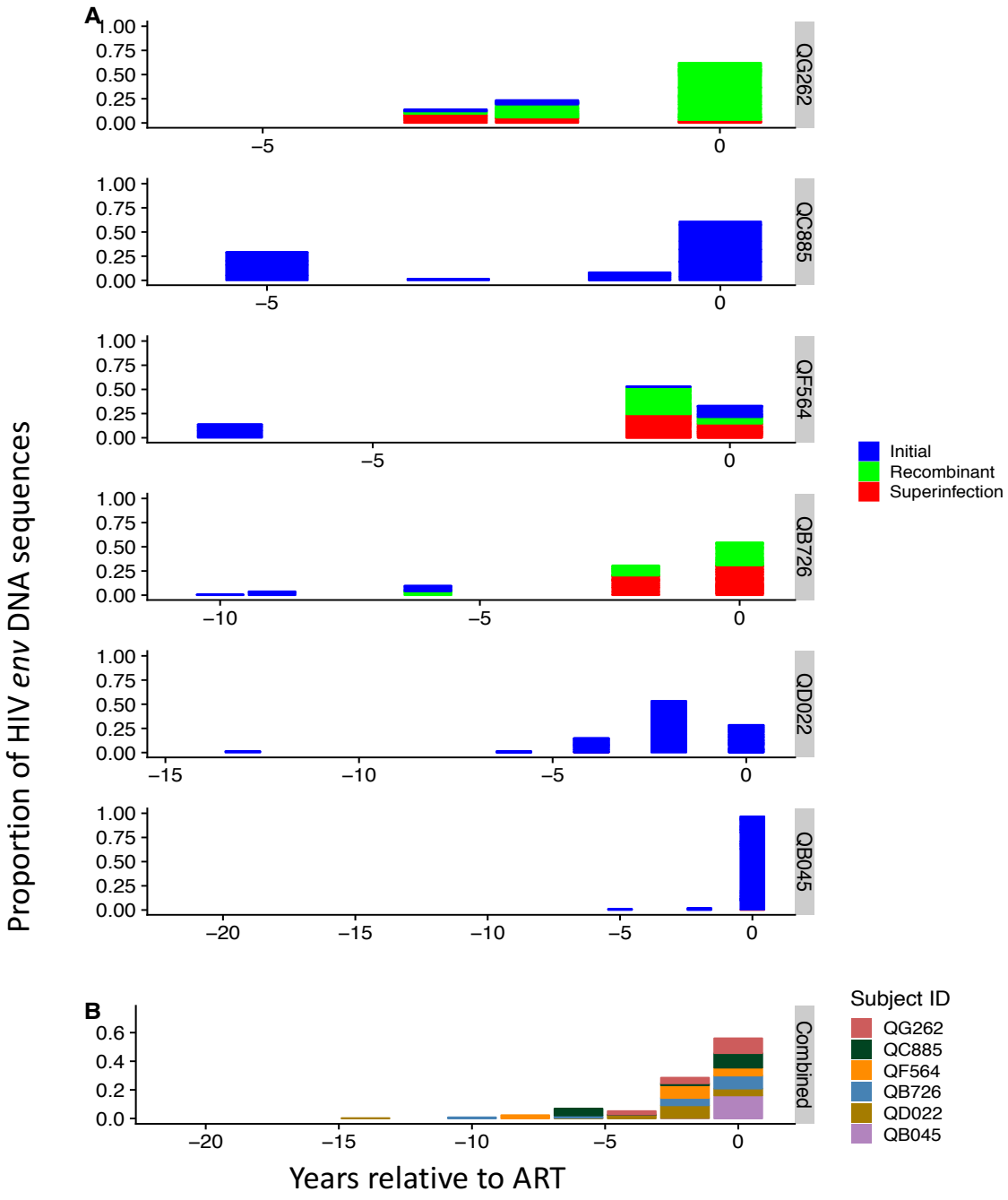


**Figure 4.7. Estimated time of HIV DNA *env* seeding in relation to ART initiation.**
Proportions of HIV DNA reservoir *env* sequences plotted against their likely time of seeding as determined by smallest pairwise distance to longitudinal HIV RNA sequences for each Subject in (A) with combined data rounded to the nearest 2-year interval in (B). In panel A blue denotes the initial viral variant, red denotes the superinfecting variant, and green denotes within region recombinants.

## 4.1 Discussion

In this study, we characterized the seeding dynamics of the HIV DNA reservoir in six Kenyan women infected with two genetically distinct HIV variants at separate time points. We deep sequenced HIV *gag* and *env* RNA from longitudinal plasma collected during acute initial infection and every ~2 years between initial infection and ART start, as well as HIV DNA from PBMCs collected >0.9 years after ART-mediated viral suppression. Our data showed that the HIV DNA reservoir can be comprised of both initial and superinfecting viral variants, which argues against the hypothesis that the reservoir is primarily seeded during early acute infection. In fact, when the initial and superinfecting viruses co-circulated over time during untreated infection, the HIV DNA reservoir was predominantly comprised of superinfecting and/or recombinant virus variants, suggesting that the majority of the HIV DNA reservoir was seeded after superinfection had occurred. In addition, pairwise distance analysis comparing HIV DNA during ART to longitudinal RNA sequences prior to ART showed that the majority of HIV DNA reservoir sequences were most closely related to HIV RNA sequences from virus circulating just prior to ART initiation. These data support previous observations by Brodin et al. and Abrahams et al. [60, 61](refs) suggesting that HIV-infected cells are rapidly turned over during untreated infection and that ART-mediated viral suppression increases the half-life of long-lived cells infected within the year prior to ART initiation.

Our methods allowed us to interrogate two different regions of the HIV genome, *gag* and *env,* both of which provided data supporting the conclusion that the majority of HIV reservoir cells are infected close to the time of ART initiation. While is some cases the viral dynamics in cell-free HIV RNA differed between *gag* and *env* (compare Figure 4.2 to Figure 4.3), this is likely reflective of recombination that occurred between these regions during active viral replication.

However, multiple HIV reservoir studies show that the majority of proviruses contain large internal deletions [51, 52], and we cannot rule out that the differences in patterns in HIV DNA sequences between regions is a result of large internal deletions. We also detected within-region recombination in at least one HIV genomic region in 5 of the 6 cases studied here. Differences in the RNA patterns between and within genomic regions typically reflect similar differences in the HIV DNA sequences between *gag* and *env* – suggesting that recombinant variants seed the reservoir.

Limitations of our study include the fact that we only sequenced HIV DNA reservoir from a single time point after ART-mediated virus suppression. However, many studies have shown reservoir stability over time with no sequence evolution and slow decay [60, 139, 140]. It is also well established that the majority of proviruses present in PBMCs during ART are replication deficient [49]. We sequenced two ~500bp regions of HIV DNA present in PBMCs, which did not allow us to distinguish between defective and replication competent virus in the reservoir. Additionally, since we did not sequence proviral integration sites, and because the regions we sequenced were short portions of the HIV provirus, we were unable to detect evidence of clonal expansion. However, our conclusions support recent findings that the HIV DNA reservoir is seeded dynamically, and predominantly just prior to ART initiation in a study of replication-competent virus [61]. Taken together, these studies suggest that novel cure strategies that target HIV infected cells within the year prior to ART initiation, may effectively reduce HIV reservoir size, increasing the likelihood of post-treatment control and a functional cure.

# Chapter 5. Conclusions and implications for HIV cure studies

## 5.1 Summary of findings

The work presented in this dissertation attempts to answer several outstanding questions regarding the seeding dynamics of the HIV latent reservoir and elucidate strategies that can be employed to limit its establishment. The HIV latent reservoir In Chapter 2 we adapted a cross-subtype qPCR assay [91] to a ddPCR format for the detection of total HIV DNA, which has been validated by other groups as a sensitive and reliable assay that may have future applications in HIV cure studies [141]. Furthermore, we developed an HIV sensitive cell line for use in the QVOA assay, which upon HIV infection secretes gaussia luciferase into the supernatant allowing for repetitive sampling of the supernatant for reactivation of latent replication competent provirus. The data in Chapter 3 is the first study to show that short treatment interruption does not significantly increase HIV DNA latent reservoir size in perinatally infected infants, and support previous studies that show early treatment limits HIV DNA latent reservoir size. Additionally, we explore the ADCC antibodies have the potential to limit HIV DNA reservoir size establishment and growth during treatment interruption, and while we do not see a strong correlation, future studies with a cohort of earlier treated infants in which passively acquired antibodies are still circulating are warranted. In Chapter 4 we estimated when the HIV DNA latent reservoir was seeded in a cohort of superinfected women and demonstrate that superinfection does not appear to influence HIV DNA reservoir seeding dynamics. Furthermore, we demonstrated that the majority of HIV DNA sequences in the HIV DNA reservoir were circulating in HIV RNA just prior to ART initiation, suggesting that the on ART half-life of the HIV DNA reservoir is much quicker than the 12 year half-life observed on ART [25]. Taken together, these studies demonstrate that the HIV

DNA latent reservoir is seeded predominantly near ART initiation, limited by early ART, and doesn't increase significantly following treatment interruption. The following chapter will reflect on the impact of these findings and their implications for the HIV cure field.

## 5.2 Barriers to HIV latent reservoir quantitation

While HIV latent reservoir quantitation methods have evolved over the past 14 years since the introduction of the QVOA, the cell based QVOA remains the gold standard assay. The biggest shortcoming of the QVOA remains the inability of current cellular activation methods to reactivate all replication competent provirus, leading to a 60 fold underestimation of reservoir size [48]. Additionally, because latent reservoir cells are so rare (1 in $10^6$ resting CD4+ T cells) the QVOA requires large volumes of blood to obtain enough cells to quantitate reservoir size. Alternative molecular based approaches require fewer cells and do not depend on a reactivation step; however, they detect both replication competent and replication deficient forms of the HIV provirus, leading to an overestimation in latent reservoir size [46]. While cell and molecular based assays each have their advantages and disadvantages, several studies have observed that measures of integrated HIV DNA by qPCR and QVOA quantitation are correlated [46, 142], and a more recent study observed correlation between measures of total HIV DNA by ddPCR and QVOA [143].

We chose to adapt an in-house *pol* qPCR total HIV DNA assay to ddPCR to measure total HIV DNA in anticipation of obtaining samples with limited cell numbers and viability. In Chapter 2 we demonstrated that our assay could detect as few as 5 copies of HIV DNA in a background of $10^6$ genomic equivalents and our assay was shown to reliably quantitate HIV DNA from different

HIV subtypes [141]. Furthermore, we used this assay in Chapters 3 and 4 to quantitate total HIV DNA from Kenyan infants and adults.

While our assay has been shown to be reliable and sensitive, it detects both replication competent and replication deficient forms of HIV provirus. Recently an exciting ddPCR multiplex assay was developed to identify proviruses that are intact by excluding proviral sequences that are likely to have hypermutations or deletions [88]. Additionally, our lab has also been developing a promising cross-subtype multiplex ddPCR assay to detect HIV provirus that is likely intact by simultaneous detection of 3 separate regions of the HIV genome. These multiplex assays will more accurately quantitate the replication competent HIV latent reservoir than assays that detect only one region, but still may overestimate the size by including genomes that have mutations in other regions that affect viral protein production. However, these assays overcome the dependency on cellular activation by the QVOA, and can be done with samples that have limited viability and cell numbers, which will contribute greatly to future HIV cure studies.

In addition to the ddPCR assay for total HIV DNA we developed a gaussia luciferase secreting tat/rev responsive cell line to be used in the QVOA. The QVOA is a labor-intensive assay that takes from 14-21 days to complete per sample and a more rapid version of the QVOA was developed that could be completed in 3-7 days [102]. This assay uses a MOLT-4/CCR5 cell line to expand virus instead of donor PBMCs and uses an RT-PCR assay to detect viral mRNAs as a read out from a limiting dilution of activated memory CD4+ T cells. The assay improves limitations to the standard QVOA but may be prone to detecting replication deficient virus. Recently an adaptation to the QVOA, known as the TZA assay was developed in which TZM-bl cells, which produce β-galactosidase when infected with HIV, were used as outgrowth and indicator cell for provirus reactivated from latently provirus [90]. While this assay is an

improvement on the QVOA as it reduces the need for outgrowth using irradiated donor PBMCs it requires destructive sampling to quantify latent reservoir size. The HIV permissive cell line we developed improves upon the TZM-bl assay as gaussia luciferase is secreted into the supernatant upon HIV infection from reactivated latent provirus. Reactivated provirus can then be identified with as little as 10µl from the supernatant of each well.

The cell line developed during this thesis has the potential to be applied to the QVOA to reduce labor and cost and studies are needed to show that this indicator cell system works within that context. Importantly it may be useful more broadly to identify better, more robust activation inducers of latent provirus. The major crux of the QVOA is the inability to reactivate latent provirus using inducers that promote cellular activation. Future assay development is needed to identify potent inducers of viral activation for use in the QVOA. Research from Razooky et al has suggested that robust proviral activation is possible using the viral protein tat [144]. Further research should be done to identify whether that mechanism of latent provirus activation can be applied to the QVOA to improve sensitivity. As the cell line developed in this thesis reduces cost and labor of identifying outgrowth viruses from reactivated latent cells, it can also be used as a screening tool to identify more potent inducers of latency reactivation in an outgrowth model.

While assays are continually being developed and improved, there are still significant limitations to overcome. CD4+ T cells comprise the major population of the latent reservoir, but HIV DNA has been detected in other cell types such as macrophages and dendritic cells [44]. Assaying these additional cell types as well as potential tissue reservoirs will be necessary to identify reductions in latent reservoir size from HIV cure studies. Additionally, latently infected cells are rare, and it is not feasible to assay all potentially latently infected cells in the body.

Therefore, barring the development of more sensitive biomarkers to identify latently infected cells, treatment interruption will be necessary to assess efficacy of HIV cure strategies.

## 5.3 HIV DNA reservoir seeding and treatment interruption

It is well established that treatment early in acute HIV infection limits the size of the HIV latent reservoir. Evidence from the Visconti Cohort suggests early treatment alone is not sufficient to promote post-treatment control [54]. However, post-treatment control is characterized by a small latent reservoir suggesting that limiting reservoir size is critical and is the current focus of many HIV cure studies attempting a functional cure by eliminating the latent reservoir.

Evaluating whether an HIV cure has been achieved will require treatment interruption, and if a small latent reservoir is indeed critical for post-treatment control, it is paramount that re-seeding of the latent reservoir be minimized if viral recrudescence occurs. The SMART trial showed that CD4+ T cell guided resumption of therapy resulted in higher incidence of morbidity and mortality [145], suggesting that a different measure should be a guide for resumption of therapy. And indeed, viral load based treatment interruption studies have been shown to be safer, and limit the depletion of CD4+ T cells [146] while providing valuable information about HIV latent reservoir dynamics. In our study of infants undergoing short treatment interruption, we demonstrated that the HIV latent reservoir did not change significantly, however the infants did not benefit from the continued decay observed in infants that did not interrupt.

Infants in our study all developed *de novo* HIV specific immune responses. Infants treated extremely early may not generate HIV specific immune responses and protection wanes as maternal passively acquired antibodies are lost. Therefore, infants treated prior to *de novo* HIV

specific adaptive immune responses may be more susceptible to increases in reservoir size during treatment interruption. Furthermore, it would be interesting to phylogenetically classify the latent reservoir prior to and after treatment interruption, to identify whether reservoir composition becomes more homogenous.

The data from Chapter 3 support other short treatment interruption studies in adults monitoring the change in the latent reservoir [78, 79], suggesting that HIV reservoir seeding can be minimized with short treatment interruption. However, it is unclear what length of treatment interruption will be necessary to identify post-treatment control. Further research is necessary to understand the mechanisms behind post-treatment control. As more individuals achieving post-treatment control are being identified, it will be important to investigate what makes post-treatment controllers refractory to viral recrudescence. Are the cells harboring latent provirus more refractory to activation signals? Recent research by Cohn et al, has suggested that latently infected cells are transcriptionally different and express factors that may suppress provirus reactivation [147]. Further research investigating the transcriptional profiles of latently infected cells from post-treatment controllers may elucidate novel mechanisms to prevent latent provirus reactivation.

However, it's unknown whether post-treatment control is lifelong. It's possible that as the immune system ages or during infection with another pathogen viral recrudescence may occur leading to renewed infection and CD4 depletion. Thus, until more is known about the mechanisms of post-treatment control, it may be necessary for individuals achieving post-treatment control to be monitored lifelong for HIV viral recrudescence.

## 5.4 Reservoir seeding prior to ART

In Chapter 4 we demonstrated that the HIV DNA latent reservoir is seeded predominantly just prior to ART initiation using samples from a cohort of superinfected women. This study suggests that the untreated decay kinetics of the HIV DNA latent reservoir is much faster than the on ART half-life of ~12 years observed by Golob et al. Untreated HIV infection causes a massive CD4+ T cell turnover of ~10^7 cells per day [148]. Resting CD4+ T cells, which harbor the latent reservoir, are killed by caspase-1 mediated pyroptosis during untreated infection [33]. Therefore, latently infected cells during untreated infection are more likely to be abortively infected and eliminated by pyroptosis than those following ART initiation.

Recent studies by Brodin et al. and Abrahams et al. have also observed that the HIV latent reservoir is seeded predominantly close to ART initiation [60, 61]. Abrahams et al. suggest that an increase in CD4+ T cells that express IL-7 receptor due to reduced inflammation as ART reduces viral load, drives effector memory T cells into a long-lived status, preserving and archiving the integrated provirus. Further studies are needed to identify the roles of both IL-7 and pyroptosis to elucidate the seeding dynamics of the latent reservoir. If the reservoir is indeed rapidly turning over prior to ART initiation, identifying mechanisms of persistence and elimination can greatly inform HIV cure strategies.

One avenue of research yet to be explored, is identifying latently infected cells during active infection. This is a challenging undertaking as it is difficult to separate latently infected cells from actively infected cells. While a biomarker identifying latently infected cells is yet to be found, Cohn et al demonstrated that latently infected cells share transcriptional profiles [147] that implicate silencing of the latent provirus. Thus, it is plausible that latently infected cells can be identified by their transcriptional profile and studied prior to ART initiation. Defining latent

reservoir dynamics prior to ART initiation may identify potential strategies to limit reservoir seeding and achieve a functional cure.

## 5.5 Conclusion

Eliminating or reducing the HIV-1 latent reservoir is a major goal of HIV cure strategies. It's been shown that the latent reservoir is remarkably stable during ART; studying latent reservoir dynamics during untreated infection guides HIV cure strategies. The studies presented in this thesis provide tools for quantitating the HIV latent reservoir and demonstrate that the HIV latent reservoir is dynamic, heterogeneous, and limited by early ART. Understanding why the latent reservoir is seeded predominantly near ART will be paramount in developing strategies to limit reservoir seeding. Combination therapies at the time of ART initiation to target and reduce seeding of the latent reservoir will be where HIV cure initiatives can have the largest impact.

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