Higher HIV-1 Env gp120-Specific Antibody-Dependent Cellular Cytotoxicity (ADCC) Activity Is Associated with Lower Levels of Defective HIV-1 Provirus

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Abstract

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During HIV-1 (HIV) infection, continued administration of antiretroviral therapy (ART) can decrease a person's viral load to undetectable levels and allow for a near-normal lifespan [1], though ART is not a cure due to a reservoir of cells that harbor latent, integrated HIV provirus capable of host immune evasion [2-7] and stochastic reactivation [8-13]. While the vast majority of HIV proviruses are defective due to large internal deletions or hypermutations [15-18], a fraction of proviruses are intact and replication competent [17, 18, 20] which contribute to the rebound of viremia that occurs upon cessation of ART, necessitating its life-long administration [2, 9, 21-23]. The majority of archived proviral sequences that persist on ART are genetically similar to those of circulating viruses at the time of ART initiation [24-26] which suggests that

factors present at this time can impact HIV reservoir dynamics, and that additional influences at this critical time for reservoir establishment may also be identified. Host immune responses such as Antibody-Dependent Cellular Cytotoxicity (ADCC), which act to clear infected cells, have been suggested to have the potential to impact reservoir size and characteristics [27-33].

Children living with HIV represent a significant fraction of people living with HIV [34], yet studies of the pediatric HIV reservoir, especially those focused on its establishment during chronic HIV infection, are relatively few compared to those in adults. The work detailed in this thesis tested the hypothesis that the ability of autologous plasma antibodies to mediate ADCC against HIV Env at the time of ART initiation inversely correlates with the size of the established HIV reservoir. This was done using samples from the Pediatric Adherence Diary study [35], the rapid and fluorometric ADCC (RFADCC) assay [36], and the Cross-Subtype Intact Proviral DNA assay (CS-IPDA) [37]. The results demonstrated a moderate, inverse correlation between HIV Env gp120-specific ADCC activity in plasma at the time of ART initiation and the level of defective ($r = -0.285$, p-value = 0.0214), but not intact ($r = -0.0321$, p-value = 0.800), HIV proviral copies that persist during ART. These findings suggest that host immune factors prior to ART initiation may impact the proviruses that persist during ART. Additionally, it adds to the mounting evidence that cells harboring defective HIV provirus may face different immune selection pressures than cells harboring intact provirus [38-40].

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Chapter 1. Introduction

Human Immunodeficiency Virus Type 1, or HIV-1 (HIV), is the causative agent of Acquired Immunodeficiency Syndrome (AIDS) which is a disease defined by severe immunosuppression and the development of opportunistic infections ultimately resulting in death if left untreated [41-47]. Since the first reported AIDS cases in 1981 [48], approximately 40 million people have died from AIDS-related illnesses [34]. Today, with continued adherence to antiretroviral therapy (ART), HIV infection has become a manageable, chronic illness by decreasing a person's viral load to undetectable levels and allowing for immune reconstitution to occur [49-55]. However, there is still no cure for HIV due to a reservoir of latently infected cells that persist on ART and cause viral rebound in the absence of continued ART administration [8-10].

Despite improvements in treatment options, there are still approximately 40 million people currently living with HIV, with over one million new infections in the year 2022 alone [34]. Of those currently living with HIV, roughly 1.5 million are children [34]. Pediatric HIV infection is unique in several aspects including multiple vertical transmission routes [56-60] such as *in utero* [58, 61, 62], intrapartum [58, 63], and via breast feeding [58, 64-67], a pre-existing, passively-acquired maternal antibody response upon HIV acquisition [68-72], accelerated pathogenesis [73-78], unique HIV reservoir dynamics [79-92], and the more frequent development of broadly neutralizing antibodies [93-98]. One thing that remains consistent between adult and pediatric HIV infection, however, is the lack of cure strategies due to the presence of the latent HIV reservoir [8-10, 99, 100].

In order to develop novel cure strategies, whether for adults or children, an understanding of the HIV reservoir and its dynamics will be required. The HIV reservoir has proven itself largely

resistant to eradication [101-108], but certain factors such as time between HIV acquisition and ART initiation [86, 109-120], and the stage of infection when ART is begun [121, 122], have been shown to impact its size and/or rate of decay. Much attention has been placed on the ability of immune-mediated mechanisms of HIV reservoir clearance [108, 123-129], with interest particularly in the possibility for cells with cytotoxic effector functions such as CD8+ T cells [130-134] and NK cells [27, 30-32, 135-137] to mediate such a clearance. Some studies have suggested that antibody-mediated effector functions such as antibody-dependent cellular cytotoxicity (ADCC) may play a role in reservoir clearance [3, 6, 28, 30, 123, 136-142]. Thus, in this thesis study, the antibody responses of children living with chronic HIV infection were evaluated for the ability to mediate ADCC against HIV Env to understand their relationship with the levels of persistent HIV provirus that are established and maintained shortly after HIV reservoir establishment upon ART initiation.

1.1 The Epidemiology of HIV-1

Today, it is estimated that roughly 85.6 million [95% CI: 64.8 – 113.0 million] people have acquired HIV infection since the start of the pandemic. Among them, approximately 40.4 million [95% CI: 32.9 – 51.3 million] people have died from AIDS-related illnesses in this time [34]. These figures suggest that the HIV/AIDS pandemic may constitute the deadliest pandemic in several centuries of recorded human history in light of the most recent mortality estimates of the 1918 flu pandemic (approximately 17 million deaths) [143, 144]. Global HIV incidence peaked between 1994 – 1996, with an estimated 3.22 million new infections (0.91/1000 people) in each of those years [145]. Since its peak, HIV incidence has been reduced by 59% but this decrease

has started to stagnate, demonstrated by the only 38% decrease in new infections between 2010 and 2022 [34].

The historical global peak of HIV incidence, and its steady decline thereafter, coincides with the doubling of monetary assistance to low- and middle-income countries for development assistance for health between 1990 and 2001 which included substantial funding to implement HIV prevention strategies [146, 147]. It also occurred at the same time as drastic improvements in treatment for HIV were developed [148] and was soon followed by the use of HIV treatment to prevent vertical HIV transmission from mothers to their infants [149-151]. In 2003, the United States President's Emergency Plan for AIDS Relief (PEPFAR) was established which greatly expanded HIV treatment access and prevention methods to people around the world and also contributed to the continued global trend in HIV incidence reduction [152]. While the global decline in HIV incidence has started to level off since 2010, the decrease in incidence among children has been maintained at a similar level to the overall decrease from incidence peak, with a 58% incidence reduction among children observed between 2010 and 2022 [34]. Despite this ongoing incidence reduction trend, in 2022 there were still approximately 1.32 million new HIV infections (0.28/1000 people) globally [145] (Figure 1.1), with 130,000 [95% CI: 90,000 – 210,000] new pediatric infections [34].

Trends in HIV/AIDS-related mortality have followed a similar trajectory to that of HIV incidence. Globally, the number of deaths associated with HIV infection peaked in 2004 with an estimated 1.90 million people having succumbed to AIDS-related illnesses in that year [153]. However, following its peak in 2004, there has been a reduction in AIDS-related deaths by roughly 69% largely due to improvements in HIV therapies around this time [154, 155] and a substantial increase in treatment access globally, expanding treatment coverage from roughly 3

million people in 2006 to nearly 22 million in 2017 [150, 156]. Again, though, this decrease has begun to diminish somewhat since 2010, with an estimated reduction in AIDS-related deaths of 51% between 2010 and 2022 [34] (Figure 1.1). In 2022 there were approximately 630,000 [95% CI: 480,000 – 880,000] AIDS-related deaths globally, of which 84,000 [95% CI: 56,000 – 120,000] were children [157]. For context, in 1993 there were approximately 620,000 AIDS-related deaths, signifying that while we have made significant progress in combatting HIV/AIDS, there is still a lot of work to be done for further improvement [153, 158]. Thus, in summary, tremendous progress has been made over the course of the HIV pandemic to decrease HIV incidence and mortality rate, though more recently that progress has somewhat decelerated. In order to reach the Sustainable Development goals to end the HIV pandemic by 2030, more work will be required to further decrease HIV transmission globally [158].

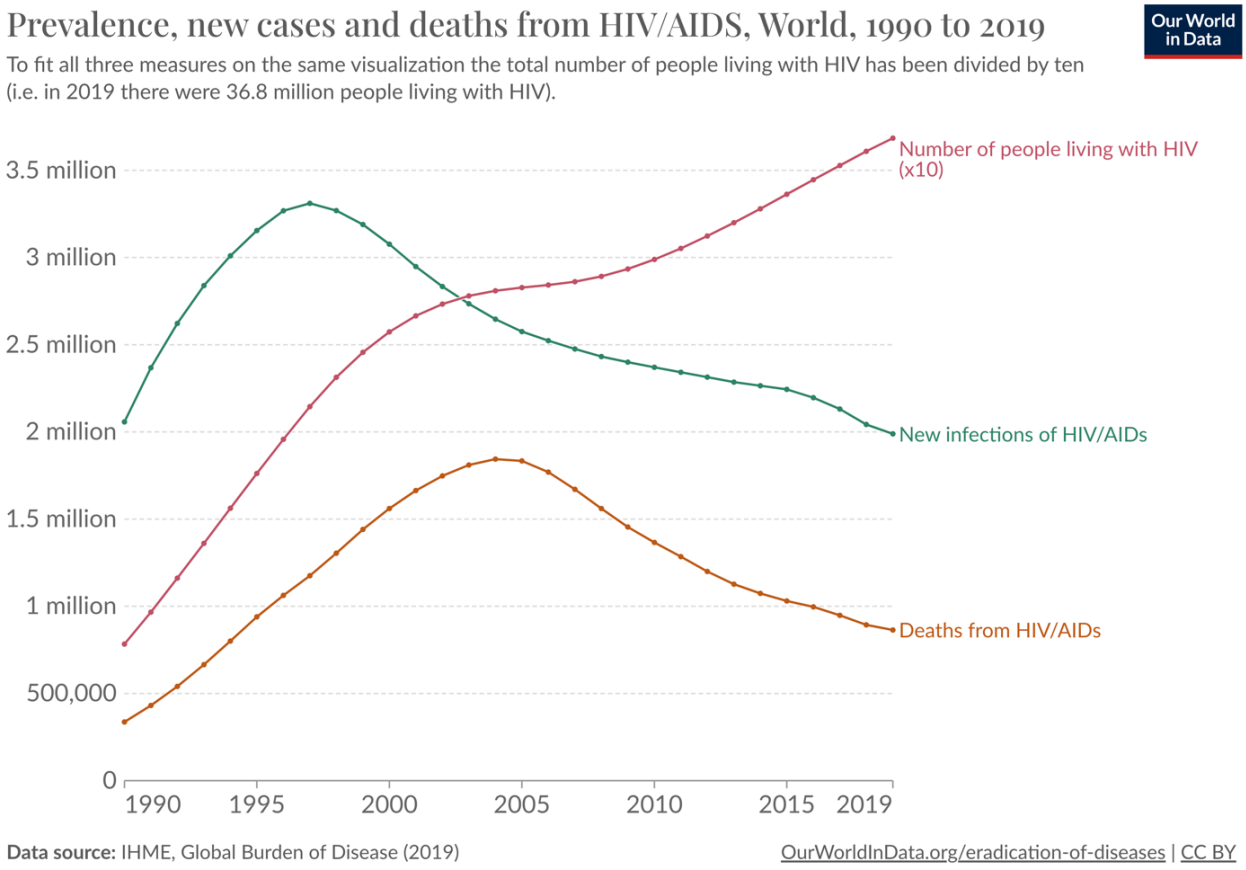

Figure 1.1 Longitudinal HIV Prevalence, Incidence, and Deaths from 1990 - 2019

The number of people living with HIV (red line), the number of new HIV infections (green line), and the number of HIV/AIDS-related deaths (orange line) plotted for each year between 1990 and 2019.

Figure 1.1 was reproduced from Max Roser and Hannah Ritchie (2023) – "HIV/AIDS" Published online at OurWorldInData.org. Retrieved from: 'https://ourworldindata.org/hiv-aids'[online resource]. Open access under the Creative Commons BY License

1.2 HIV-1 Transmission and Pathogenesis

The transmission of HIV represents a genetic bottleneck where typically a single transmitted/founder (T/F) virus that was previously circulating amongst a quasispecies within the transmitting host establishes infection in a new host [159-163], though there is accumulating evidence to suggest that in some instances multiple founder viruses can establish infection [164, 165]. The most common route of transmission for HIV is sexual transmission, though intravenous drug use and exposure to contaminated blood products are also exposure events that can lead to HIV acquisition [34]. Vertical transmission of HIV to infants, which accounts for nearly all pediatric HIV cases, can also occur through multiple mechanisms including *in utero*, intrapartum, or postnatally via breast feeding [56, 61, 166-170].

The establishment of HIV infection begins with an acute infection phase represented by remarkably high viral replication and rapid dissemination of the virus [171-176]. In adult cases, this occurs in the absence of a pre-existing humoral immune response. The virus quickly spreads in the gut-associated lymphoid tissues (GALT) and virus-induced cytopathic effects cause infected cells to die, resulting in a substantial depletion of CD4+ T cells in the GALT [171, 177-184]. This causes a subsequent increase in immune activation to promote cellular turnover to replenish what is lost [185-188]. It also leads to a loss of mucosal integrity in the intestines, inducing microbial translocation into the bloodstream [181, 182, 184, 189-191] (Figure 1.2). The virus then spreads to the peripheral lymphoid tissue where it establishes high levels of viremia [181].

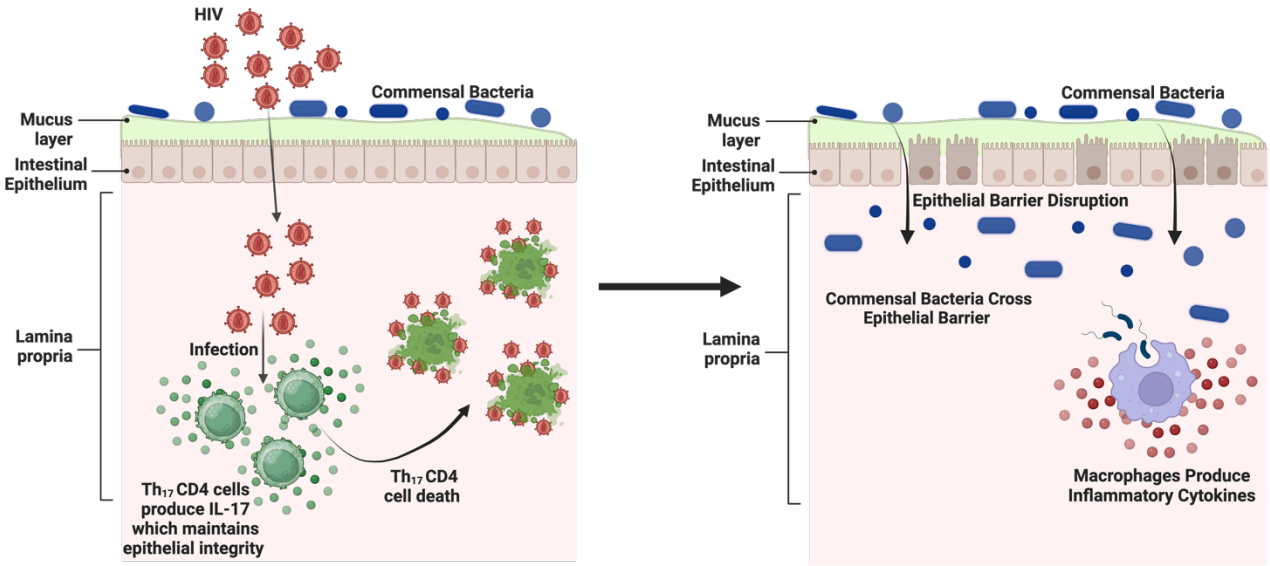

Figure 1.2 Depiction of HIV-1-Induced Gut Permeabilization

Visual representation demonstrating how infection of Th_{17} CD4+ T cells by HIV-1 can lead to intestinal permeabilization and subsequently chronic immune activation. On the left side of the arrow, an image depicts HIV entering the gut, infecting Th_{17} CD4+ T cells, and causing them to die. On the right side of the arrow, an image depicts how lipopolysaccharides derived from commensal bacteria in the gut can cross into the lamina propria via intestinal permeabilization and result in the production of inflammatory cytokines.

Figure 1.2 was created using BioRender.com

Roughly 2-4 weeks after HIV acquisition, most people develop acute HIV syndrome which is associated with a high viral load, flu-like symptoms including fever and headache, and lymphadenopathy [192-194]. Around this same time viral load typically peaks, followed by several months of gradually declining viremia until reaching a relatively stable viral load setpoint (VLSP) which has been reported to correlate with the rate of disease progression [195-199]. Following this period is the establishment of the chronic infection phase of HIV infection [176, 200] (Figure 1.3).

Untreated chronic HIV infection can typically last for 5-10 years in adults before advancing to AIDS [47, 198, 200]. As chronic infection progresses, an average decline of 50-100 CD4+ T cells/µl of blood per year occurs [201], with this rate impacted by factors such as viral load set point and the infecting virus subtype [47, 196, 198, 201] (Figure 1.3). As CD4+ T cells decline and a person's immune response continuously tries to keep up with the persistently circulating virus, pathogenesis associated with immune dysfunction such as hypergammaglobulinemia ultimately results which can lead to co-morbidities such as autoimmune disorders [202].

This immune dysfunction includes impaired macrophage utility, likely both because they are cellular targets for HIV [203-206] and because they are persistently activated during chronic infection [104, 135, 207]. This results in the impaired ability for macrophages to phagocytose or clear intracellular pathogens [206, 208] in addition to contributing to chronic immune activation levels that directly contribute to disease pathogenesis and comorbidities [104, 135, 207, 209, 210].

The chronic antigen stimulation and immune activation faced by the host immune response in its attempts to control HIV infection is exacerbated even further by the microbial translocation that results from the intestinal barrier disruption during infection [182, 184, 189-191, 211]. The introduction of lipopolysaccharides (LPS) into the bloodstream from the intestines causes the sustained secretion of cytokines and chemokines, as LPS is a potent immune stimulator [183, 189, 191, 207, 211]. LPS levels in the blood are elevated throughout chronic infection, and when combined with viral antigenic stimulation, results in the exhaustion of various lymphocyte populations as they face persistent increased turnover and differentiation [191, 212, 213]. Immune cells therefore experience a progressive loss in polyfunctionality and proliferative capacity, ultimately diminishing their potential to regenerate and promoting the progression of HIV infection to AIDS [212, 214]. This immunologic dysfunction is far reaching, as B cells and

Natural Killer (NK) cells are also negatively impacted by this chronic antigenic stimulation, signifying the overall immune dysregulation caused by untreated, chronic HIV infection [215-220].

Thus, the immunosuppressive, pathogenic nature of HIV disease is thought to be dependent on chronic immune activation. Further evidence for this is implied by the fact that there is an absence of immune activation in nonpathogenic Simian Immunodeficiency Virus (SIV) infection despite the presence of high viral loads [221-223]. This contrasts with HIV infection, where sustained viral infection and immune stimulation ultimately leads to the inability of CD4+ T cells to replenish themselves, causing their gradual depletion [45, 224-226]. Once sufficiently depleted, the dearth of CD4+ T cells causes immunodeficiency and progression to AIDS which is defined by having a CD4 cell count < 200 cells/µl or the presence of an AIDS-defining condition regardless of CD4 cell count [227, 228]. AIDS-defining illnesses include, but are not limited to, Kaposi sarcoma, Candidiasis (fungal infection) that is typically oropharyngeal, recurrent pneumonia, cytomegalovirus disease and/or retinitis, and chronic ulcers in addition to others [46, 227-229]. If left untreated, these AIDS-defining illnesses typically result in death [46, 47, 228, 229].

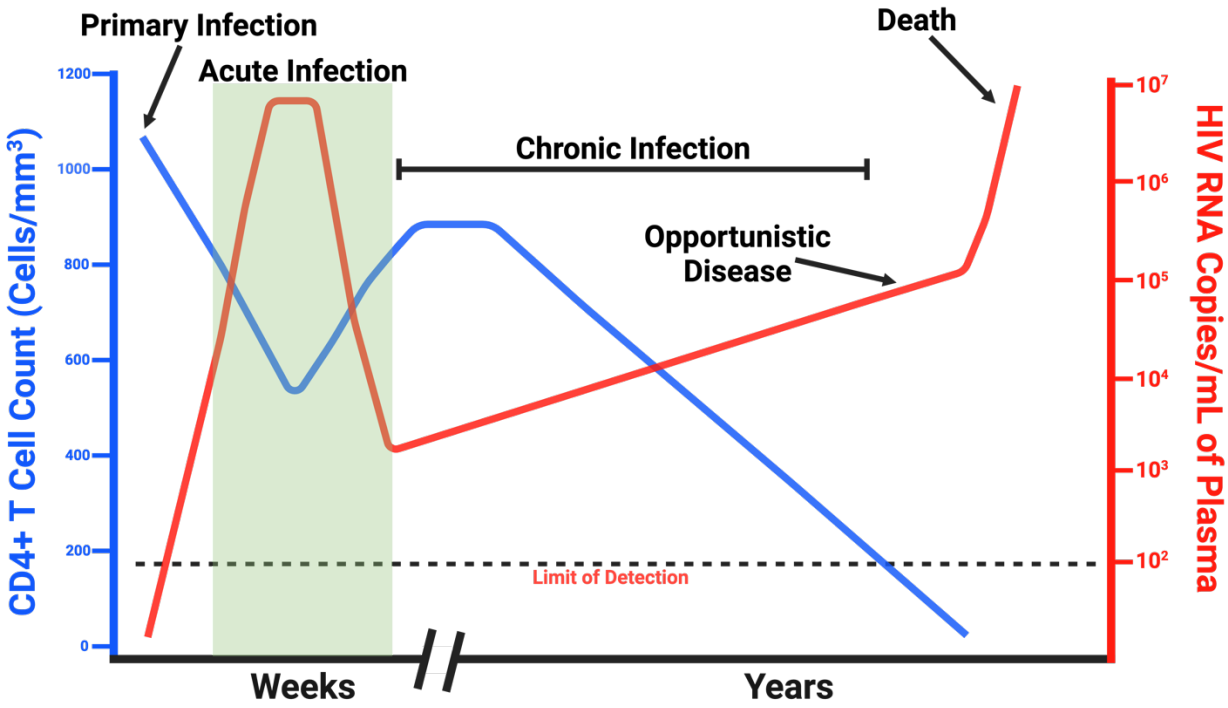

Figure 1.3 Typical Time Course of Untreated HIV-1 Infection

Typical disease progression of untreated HIV-1 infection, with the left Y-axis and blue lines representing CD4+ T cell counts (cells/mm^3), and the right Y-axis and red lines signifying plasma viral load (RNA copies/mL). The X-axis is time since seroconversion. The "Limit of Detection" is referencing the clinical detection of viral RNA.

Figure 1.3 was created using BioRender.com

1.3 Pediatric HIV-1 Infection

The transmission and pathogenesis of HIV in the setting of pediatric HIV infection are considerably different compared to adults, including time to developing AIDS and typical transmission routes. Among children living with HIV, nearly all cases are a result of vertical transmission from the child's mother, with three different possible transmission routes: *in utero* (risk of infection: 5-10%) [56, 58, 61, 62], intrapartum (risk of infection: 10-20%) [56, 58, 63], or via breast feeding (risk of infection: 5-15%) [56, 58, 64-67], with the risk of transmission directly correlated with maternal viral load and indirectly with maternal CD4 count [56, 59, 60, 64, 170,

230-234]. Transplacental HIV transmission typically occurs late in the third trimester [235, 236] and provides the best chance to quickly detect infection at point of care to allow for the infant to begin ART soon after infection establishment/birth [58, 237-239]. However, if *in utero* transmission occurs during late gestation, a recently established HIV infection may not yet be able to be detected at birth due to insufficient viral load [240]. Intrapartum transmission is the most common route of vertical transmission and is most likely a result of mucosal exposure to infectious bodily fluids during childbirth [241-247]. Breast milk transmission occurs as a result of frequent, daily potential exposures to breast milk that contains both free virus and infected cells [57, 248-258]. For babies who are born preterm, the risk of vertical transmission via breast feeding or during childbirth is doubled compared to those born at term [259]. Correlates of vertical HIV transmission are discussed in more detail in section 1.9.

The pathology of untreated pediatric HIV infection is particularly heinous. For infants living with HIV, approximately 30% will progress to AIDS and die within their first year of life, with approximately 50% suffering the same fate by age two [77, 78], and up to 80% by age five [73]. This rapid progression to AIDS in infants is facilitated by an expeditious rise in viremia following infection. Within months of vertical transmission, an infant's viremia typically expands ten-fold, usually to levels higher than is ordinarily seen in adult HIV infection [260-263]. Viral load commonly reaches over 100,000 copies/mL in this timeframe with the ability to reach over one million copies/mL, followed by a slow decline not reaching VLSP until around age five if the child survives to see this age [262, 264].

Similar to what is observed with transmission risk, the prognosis of perinatal HIV infection varies by transmission route, as well. In one study that measured mortality of infants in Zimbabwe, for infants who experienced vertical transmission *in utero*, the median survival time

was only 208 days [265]. Infants who experienced intrapartum vertical transmission demonstrated a nearly doubled median survival time of 380 days, though still only roughly one year [265]. Infants with vertical transmission via breast feeding typically lived the longest, with a median survival time of over 500 days [265].

The exact reason for the increased pathology in pediatric HIV infection compared to that in adults is unknown. However, the distinct features of the developing early life immune system may play a role [266]. For example, perinatal and early life development requires the immune system during these stages of life to be largely tolerogenic as not to make detrimental immune responses to maternal antigens or to the bacteria that are colonizing multiple anatomical compartments during microbiome establishment [267]. This results in a developing immune system to be skewed towards a T_H2 response which is more anti-inflammatory and which comes at the expense of a T_H1 response. A T_H1 response is more suited towards combating extracellular microbes such as bacterial and viral infections [268].

Knowing that HIV disease pathogenesis is largely mediated by inflammation, one might expect the anti-inflammatory nature of the early life immune system to result in decreased pathogenesis. However, this is not the case. By focusing on developing a tolerogenic immune environment in early life, to skew towards a T_H2 response infants generally have a higher concentration of T_{Reg} cells which relies on immune defenses largely mediated by T_H17 cells [244, 267]. These T_H17 cells are found in high concentrations in the gut and are important for maintaining intestinal epithelial integrity [177, 178, 269]. However, these cells are also particularly permissive to HIV infection and may result in higher levels of infected cells and increased intestinal permeabilization [171, 180, 244] (Figure 1.2). Thus, the unique features of the

early life immune system may be exploited by viral replication mechanisms to facilitate the unique features of HIV pathology observed in children [266, 268].

1.4 HIV-1 Life Cycle

HIV is an enveloped virus approximately 100-120 nanometers in diameter surrounded by a lipid membrane derived from the membrane of the host cell from which it originated [270]. There are two viral glycoproteins that span this lipid membrane, the outer surface glycoprotein (SU) and the transmembrane glycoprotein (TM) [5, 271]. Inside the virion, the inner surface of the viral membrane is lined by matrix proteins (MA), with capsid proteins (CA) forming a cone-shaped core [270, 272, 273]. Inside this core contains two identical molecules of positive sense genomic viral RNA [274, 275] that are associated with nucleocapsid proteins (NC), together forming a ribonucleoprotein complex [276-279]. The virion additionally contains three viral enzymes: protease (PR) [280, 281], reverse transcriptase (RT) [281, 282], and integrase (IN) [283], in addition to the accessory protein Vpr [284] (Figure 1.4).

The viral genome is approximately 9.5 kilobases in length containing two identical long terminal repeats at the 5' and 3' ends [274, 281, 285, 286]. The genome encodes for three major polyproteins common to all retroviruses: *gag*, *pol*, and *env* [281, 287]. Additionally, it encodes for two regulatory proteins Tat and Rev, and the accessory proteins Vif, Vpr, Vpu, and Nef [287].

The viral *gag* gene contributes the structural proteins essential for virion assembly which are the MA, CA, and NC proteins [288-290]. The MA protein is important for viral assembly, moving Gag polyproteins to the cellular plasma membrane and helping to incorporate the SU and TM glycoproteins into nascent virions [291, 292]. The CA protein is a 24 kDa protein, known as p24 gag, that makes up the viral core within the virion that contains the packaged viral RNA [270,

272, 273]. The NC protein binds to a packaging signal present within the viral RNA genome, delivering it to the assembling virion. NC also non-specifically binds to single-stranded nucleic acids, coating the viral RNA within the core of the mature virion [276, 278, 288].

The viral *pol* gene encodes the PR, RT, and IN enzymes that are packaged within the virion and has considerable overlap with the *gag* gene in the viral genome, with its translational reading frame -1 with respect to that of *gag* [293]. Due to ribosomal frameshifting, a Gag-Pol fusion protein is produced during translation with a frequency between 5-10% [293-295]. The PR enzyme is produced by cleaving itself from the Gag-Pol polyprotein and is responsible for also cleaving the RT and IN enzymes from this polyprotein [296]. The RT enzyme is capable of multiple catalytic activities including DNA polymerase initiated from either an RNA or DNA template and RNase H activity capable of degrading RNA strands in DNA:RNA hybrid molecules [297, 298]. It uses these abilities to reverse transcribe viral RNA into a DNA provirus [299-301]. The IN enzyme also exhibits multiple functions, including the ability to bind to DNA, DNA endonuclease activity, DNA integration, and recombination [302-305]. It is also responsible for incorporating the HIV provirus into the genome of its host cell [304, 305].

Finally, the third major gene found in the HIV genome is the *env* gene. *Env* encodes the SU and TM glycoproteins that are found on the surface of the HIV virion and are involved in viral entry and fusion to the host cell [306, 307]. The initial Env protein synthesized from the viral genome is a 88 kDa precursor [308] that, upon translocation into the rough endoplasmic reticulum, is glycosylated and folded into its tertiary structure [309-311]. This processed protein has a mass of 160 kDa, being commonly referred to as gp160 [310, 312]. Env is very heavily glycosylated, with glycosylation accounting for approximately half of its molecular weight [312-314].

The gp160 protein is cleaved by endogenous furin in the Golgi to produce a noncovalently linked glycoprotein trimer consisting of the 120 kDa SU (gp120) and 41 kDa TM (gp41) subunits [315-317]. The Env trimer is then transported to the plasma membrane of its infected host cell to be incorporated into viral particles [311]. The SU protein is exposed to the extracellular environment thus facilitating binding to the host cell and determining viral tropism [306, 307]. HIV Env is also immunogenic and the sole target of neutralizing antibodies that its host develops during untreated infection [318, 319]. The TM protein anchors the protein complex to the virion lipid membrane and is essential for mediating fusion between the viral and host cell membranes after receptor binding [311, 320-325]. An individual HIV virion typically displays very few Env trimers on its surface, with reports ranging from 2 – 14 Env trimers observed per virion which is sufficient to mediate infection of a host cell while also minimizing exposure for recognition by the immune system [326-328].

The two regulatory proteins Tat and Rev play crucial roles in viral replication and viral gene expression. The Tat protein is important for the processivity of transcribing polymerases and can also enhance viral transcription initiation [329]. Rev binds to the nuclear export protein CRM1 and is responsible for transporting singly spliced and unspliced viral RNA transcripts from the nucleus to the cytoplasm for translation and packaging into assembling virions [330, 331].

The HIV viral genome also encodes four accessory proteins: Nef, Vpu, Vif, and Vpr [3, 7, 284, 332-335]. The Nef protein downregulates cell surface expression of both CD4 and MHC class I of infected host cells [104, 131, 336-339]. In doing so, Nef decreases interactions between CD4 molecules and the Env proteins on budding virions that are both present on the cell surface, promoting the release of newly assembled viral particles that may otherwise have been stuck to host CD4 [140, 338, 340]. The downmodulation of MHC Class I on the cell surface promotes

immune evasion of infected cells by reducing recognition of presented viral antigens by cytotoxic T lymphocytes [4, 131, 339, 341]. The Vpu protein is an antagonist of the host cell protein tetherin which is a protein that prevents the proper release of accumulated virions on the cell surface [342-344]. Like Nef, Vpu also downmodulates CD4 expression on its host cell's surface [6, 141, 340, 345]. Thus, along with Nef, Vpu promotes the budding of mature virions and increases the rate of virus release from its host cell [6, 141, 340, 346]. The accessory protein Vif is involved in counteracting the host's innate defenses from viral infection by degrading the host restriction enzymes that can be packaged in virions that introduce hypermutations into the viral genome during reverse transcription [332, 333, 347-349]. The Vpr protein induces cell cycle arrest, enhances viral replication, and dampens the interferon response associated with HIV infection [175, 203, 334, 335, 350, 351].

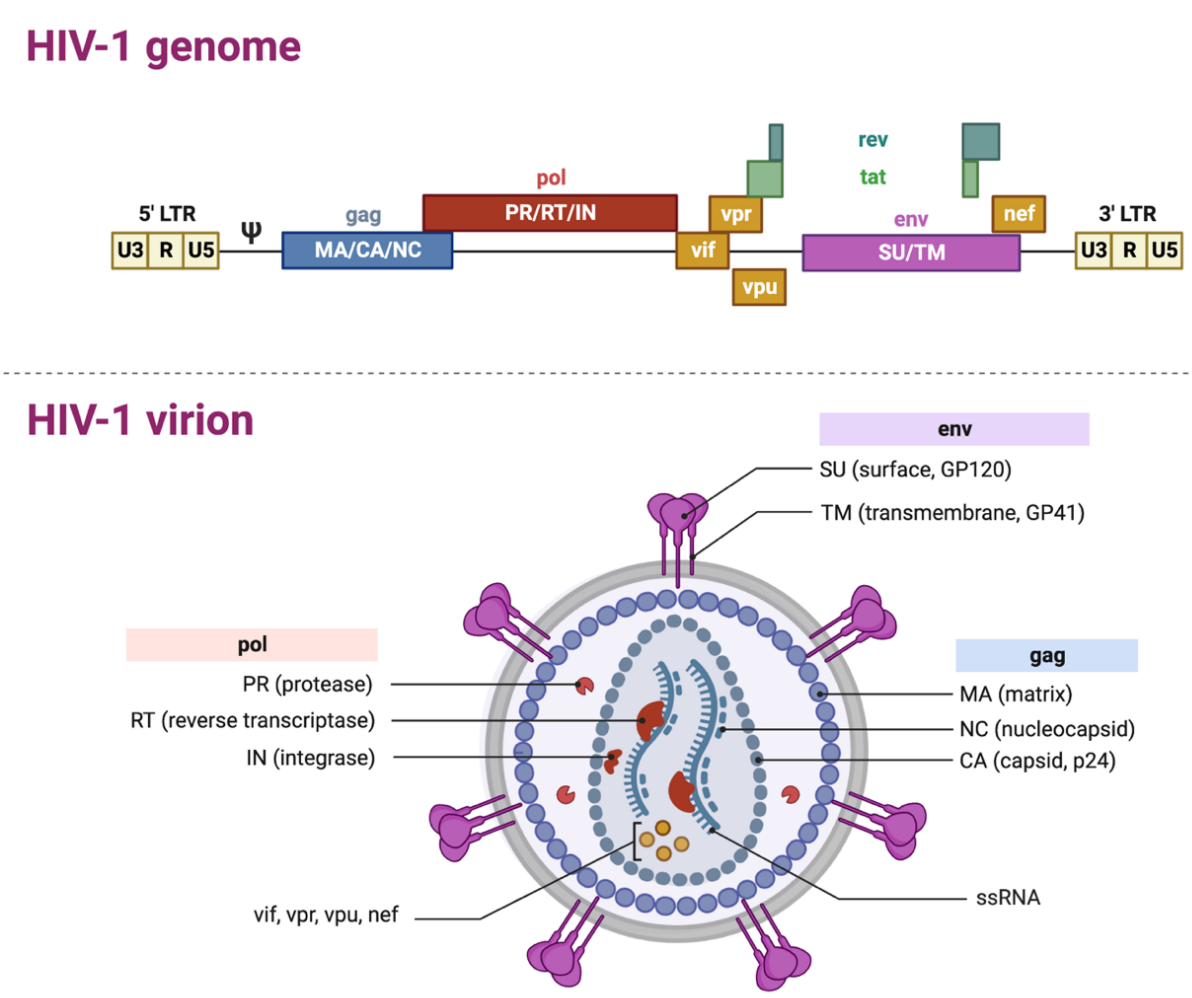

Figure 1.4 HIV Genome and Virion Composition

Visual representation of the organization of the HIV genome (top), and an HIV virion and its components (bottom)

Figure 1.4 was reproduced from "HIV-1 Genome and Structure," by BioRender.com (2023)

Together, these proteins work in tandem to facilitate the HIV viral life cycle. HIV primarily infects CD4+ T cells but can also infect other immune cells such as macrophages and dendritic cells [204, 352, 353]. Its life cycle begins when a virion encounters the viral receptor CD4 that is expressed on a host cell's surface and binds to it via the SU glycoprotein [306, 307, 311]. Importantly, for the virus to be internalized, SU must also bind to a chemokine co-receptor typically either CCR5 [354] or CXCR4 [355-358]. Upon binding, a conformational change in the TM protein triggers the fusion of the virus and host cell membranes, releasing the viral core into the cellular cytoplasm [311, 320-322].

Here, with tRNA already bound to the primer binding site approximately180 nucleotides away from the 5' end of the viral RNA genome, the RT enzyme begins the process of converting the viral RNA into viral DNA [297, 359, 360]. This process begins with tRNA directing RT to synthesize a small DNA segment towards the 5' end of the viral RNA genome resulting in a short-lived RNA:DNA hybrid molecule [297, 359, 360]. The RNase H activity of RT soon degrades the RNA portion of this complex. The short DNA fragment that remains then binds to the corresponding identical segment at the 3' end of the second copy of viral RNA, with RT synthesizing minus-strand DNA until it reaches the primer binding site at the 5' end of the viral RNA [359, 361]. Again, RNase H activity digests the RNA portion of the resulting RNA:DNA hybrid, leaving a small segment at the 3' end of the RNA where the DNA segment was bound that is then used as a primer to direct the synthesis of plus-strand DNA which involves copying a portion of the bound tRNA [298, 359, 361].

Following plus-strand synthesis, the bound tRNA at the 3' end of this plus-strand is removed via further RNase H activity, exposing the primer binding site and allowing for the hybridization of this site to the corresponding identical site on the minus-strand DNA [298, 359, 361]. The HIV

provirus is then fully reverse transcribed after the RT enzyme fills in both strands of DNA, resulting in a double-stranded DNA viral genome with complete LTRs at both ends [323, 359]. Once in its double-stranded DNA form, the IN enzyme removes two nucleotides at the 3' end of both strands of viral DNA and performs a similar cleavage in the host cell genome, introducing overhangs that allow for the integration of the viral DNA 3' overhangs into the cellular DNA 5' overhangs [302, 303]. Cellular repair machinery then fills in the resulting gaps and the provirus is forever integrated into its host cell and all of its future daughter cells [302-305, 362].

The proviral 5' LTR promotes the transcription of the viral genome via host cell RNA polymerase II, first transcribing short, spliced RNA segments that encode for Tat, Rev, and Nef [329, 363, 364]. The transcription of Tat results in a feedback loop where Tat production promotes increased expression from the HIV LTR, causing levels of Rev to increase accordingly [329, 363, 364]. Rev binds to the Rev response element (RRE) in the viral genome and transports unspliced viral RNA from the nucleus to the cytoplasm, resulting in the production of Gag/Gag-Pol polyproteins and the Env glycoproteins [330, 331, 364, 365]. Through auto-processing events, PR cleaves itself from the polyprotein where it can then liberate MA, CA, and NC so they can act as stand-alone proteins [366]. Having been cleaved from the polyprotein, the MA, CA, and NC viral enzymes become active and induce structural rearrangements of the developing virion which aids in the assembly of virion components at the cellular membrane and promotes viral infectivity [367-371]. Finally, Vpu and Nef promote the release of newly formed virions, allowing the process to start all over again [6, 141, 372] (Figure 1.5).

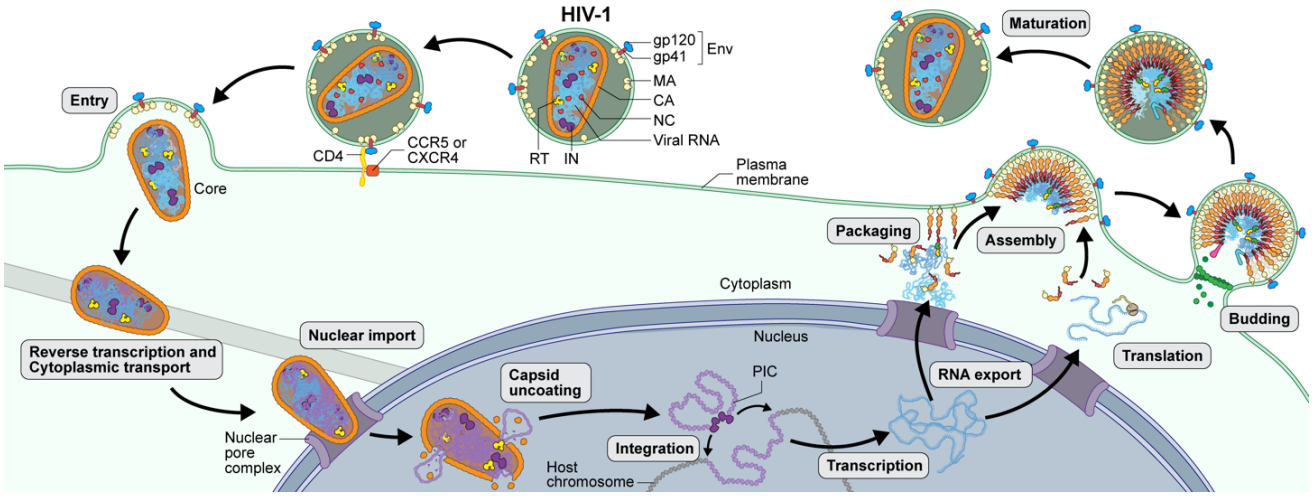

Figure 1.5 The HIV Viral Life Cycle

Visual representation of the HIV viral life cycle starting with viral attachment (left) and ending with the budding of a mature virion (right)

Figure 1.5 was reproduced from Science of HIV, "The HIV Life Cycle," scienceofhiv.org/wp/life-cycle/ Open access under the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License

1.5 HIV-1 Evolution and Diversity

A defining feature of the HIV viral life cycle, as a member of the *Retroviridae* family of viruses, is the reverse transcription of viral RNA into DNA using its reverse transcriptase enzyme [323]. Importantly, the HIV RT enzyme is characteristically error-prone which results in a high rate of mutations of the viral genome. Due to RT's lack of proofreading ability, an estimated 1-10 mutations are introduced per genome per viral replication cycle which contributes to the significant diversity that is distinctive of circulating HIV strains [373-381]. An additional mechanism that contributes to the extensive diversity of HIV occurs when a host cell is infected by two distinct viral variants, with the resulting viral progeny being heterozygotes that contain one genomic copy from each infecting strain [380, 382-384]. When this heterozygous virus

infects a new host cell, during reverse transcription, RT can transfer between the two RNA templates and produce a provirus that is a mosaic of the genomes of the co-infecting variants [373, 380, 382-385].

Changes to the viral genome during reverse transcription can also be caused by host restriction factors such as the APOBEC family of cytidine deaminase enzymes that can introduce hypermutations or large internal deletions into the proviral genome [16, 92, 332, 333, 347, 349, 378, 386-389]. It is well-established that these mechanisms of frequent mutations cause the vast majority of proviral sequences that are produced during HIV infection to be defective, meaning they are unable to produce replication competent virus capable of establishing novel infection [16, 18, 38-40, 349, 390, 391]. This is in contrast to intact proviruses which typically contain a functional HIV genome capable of producing replication competent, infectious virions [18, 39, 108, 390, 392-396]. Although mutations in the viral genome are often deleterious, occasionally they can also be beneficial for the virus as they allow for the rapid development of mutations capable of evading immune defenses or drug interventions [319, 374, 397].

Indeed, because the initial antibody response (described in section 1.8) to HIV is non-neutralizing, there is a lack of selection pressure on HIV *env* among replicating virus early in infection [163, 319]. This lack of selection pressure from the host adaptive immune system, paired with the high mutation rate of HIV, results in the evolution of viral sequences in a largely random fashion during early acute HIV infection, exhibiting a Poisson distribution of mutations [163, 398-400]. The virus is capable of escaping neutralizing antibody responses once they do eventually develop via mechanisms such as single amino acid substitutions [401-403] and insertions and deletions (indels) [404-406] in Env that are induced by the previously described processes of diversification. Therefore, during untreated HIV infection, the circulating viruses

are maintained as a continuously evolving quasispecies population with constant diversification [50, 407-409].

This perpetual diversification has resulted in the development of an array of distinct circulating HIV variants classified into four main groups, which can be further subdivided into different subtypes present within a group [377, 410, 411] (Figure 1.6). The four groups of circulating HIV include groups M (Major), O (Outlier), N (Non-M, Non-O), and most recently P [377, 410, 411], each representing a distinct zoonotic spillover event of SIV from nonhuman primates, with HIV group M the only group that has resulted in pandemic spread [14, 19, 412]. Viruses within group M are further divided into nine distinct subtypes based on genetic distances and phylogenetic clustering [14, 19, 413, 414] (Figure 1.6). These subtypes are classified as: A, B, C, D, F, G, H, J, or K [19, 411, 414], each of which originated as a result of founder effects when HIV was introduced to a certain geographic region and further diversified within that specific region [377]. Genetic variation between subtypes typically ranges between 25-35% [413-415]. In addition to the nine distinct subtypes within HIV group M, circulating recombinant forms (CRFs) have also been identified. A CRF constitutes a mosaic genome consisting of genomic regions from distinct subtypes and must be isolated from at least three people who are not epidemiologically linked and fully sequenced to be classified as a CRF. The two most common CRFs are CRF01_AE and CRF02_AG [19, 413, 415].

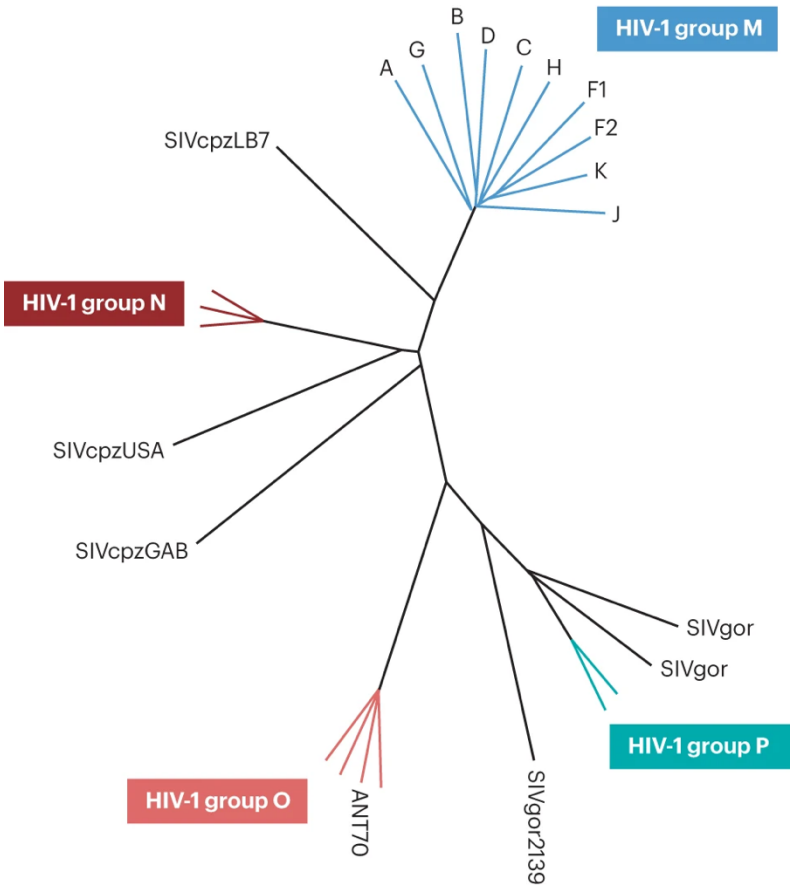

Figure 1.6 Phylogenetic Representation of HIV Origins and Subtypes

Phylogenetic analysis of the SIV origins of currently circulating HIV groups, in addition to the genetic relatedness of each group's subtypes. Figure 1.6 was reproduced from [14] with permission, Springer Nature license number: 5670260280494

Globally, the most prevalent circulating HIV subtypes are subtype C (23%), A (16.7%), and CRF01_AE (9.5%) [19]. Analyzing HIV subtype distribution by geographical region, CRFs predominate in West and Central Africa (CRF02_AG), the Middle East and North Africa (other CRFs), and Asia and the Pacific (CRF01_AE) [19]. In North America, West and Central Europe, Latin America, and the Caribbean, subtype B is the predominant circulating subtype, whereas subtype A is the predominant strain identified in Eastern Europe and Central Asia. In Eastern and Southern Africa, where the greatest burden of disease for HIV is located, subtype C is the predominant circulating strain (Figure 1.7) [19]. The existence of such a wide array of circulating HIV subtypes is a testament to how widespread HIV infection continues to be and adds to the importance of preventing HIV transmission chains. This can be most effectively achieved

through the expansion of access to treatment for HIV infection which significantly reduces HIV transmission risk [416].

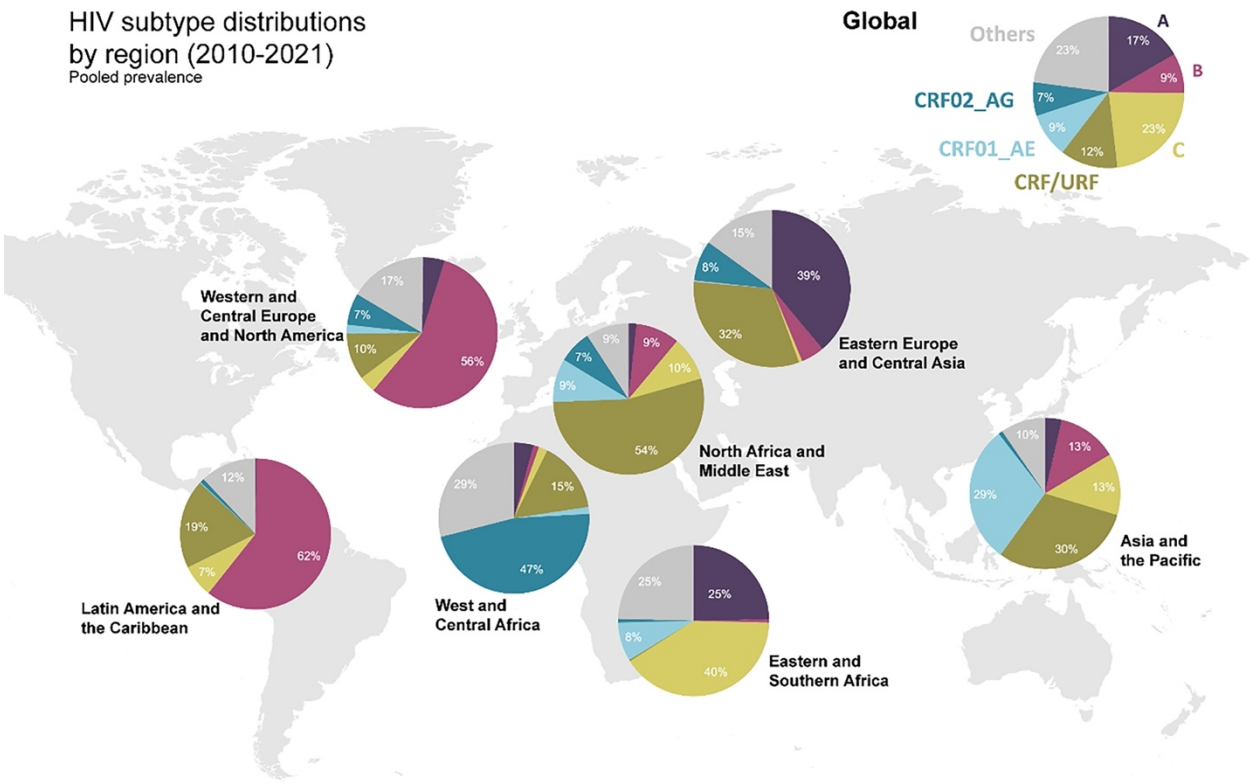

Figure 1.7 Global HIV Subtype Distribution (2010 – 2021)

Map data visualization showing the proportion of circulating HIV subtypes within each global geographical region

Figure 1.7 was reproduced from [19], Open access under the Creative Commons BY License

1.6 Treatment for HIV-1 Infection

In the over four decades since cases of HIV were first reported [48], many options for treatment of HIV infection have been developed including six major drug classes [417, 418]. The first class of drugs developed to treat HIV were Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs) [419-421]. This class of drugs works by inhibiting activity of the RT enzyme due to their structural similarity to nucleosides and nucleotides such as dATP, dCTP, dTTP, and dGTP and compete with these natural nucleotides for incorporation by RT into the viral DNA genome [420]. Drugs of the NRTI class typically compose the backbone of modern first-line antiretroviral therapy (ART) [417, 422]. Another class of drugs, nonnucleoside reverse transcriptase inhibitors (NNRTIs), work similarly by inhibiting the RT enzyme from completing the conversion of viral RNA into DNA [423]. Protease inhibitors are an additional class of drug compounds that work by mimicking the substrates of the PR enzyme by attaching to its active site and disrupting its activity, preventing the creation of functional virions [424].

Another, lesser used category of ART drugs is the category of entry inhibitors [425] which prevent virus binding [426] or fusion [427] to susceptible host cells. The integrase inhibitor drug class is a much more commonly used class of drugs in first-line treatment because of their high efficacy, tolerability, and genetic barrier to resistance [422, 428]. These drugs function by inhibiting IN enzyme activity by binding to the enzyme's active site, blocking the viral DNA from properly interacting with IN and preventing integration of the provirus into the host cell genome [428, 429]. The newest addition to the arsenal of available ART options was just approved in late 2022 with the drug lenacapavir, the first and only CA inhibitor [372, 418, 430]. It works by binding directly to the CA protein thus interfering with both the nuclear transport of proviral DNA, as well as the assembly of new virions [418, 431].

While there are many individual drugs available for ART, in 1995 a clinical trial combining a protease inhibitor with two NRTIs was the first to demonstrate that a combination ART regimen could reduce a person's viral load to undetectable levels and avoid the development of drug resistance and virological failure, forever revolutionizing the treatment of HIV [148]. For 10 years after combination therapy was first proven to be effective, ART regimens consisted of taking multiple pills sometimes multiple times a day which was often cumbersome for their users [154]. It wasn't until 2006 that the first single-tablet regimen consisting of two NRTIs and one NNRTI became available [154, 155]. Today, single-tablet regimens optimized for minimum side effects and high genetic barriers to resistance are standard first-line treatment, typically consisting of two NRTIs and one integrase inhibitor [422]. Once-daily single-tablet regimens are simpler and easier than previous multi-pill regimens, significantly improving drug adherence [154, 422].

When a person begins ART, generally two phases of HIV RNA decay occurs [432-435]. The first phase is typically the shortest, usually occurring within the first 5-10 days after ART initiation [432, 433, 435]. This period is defined by the rapid clearance of viral RNA through the elimination of actively infected CD4+ T cells which generally have a half-life of approximately one day [432, 434-436]. Following the clearance of actively-infected cells, the second phase of HIV RNA decay occurs which is slower and continues until an undetectable viral load is achieved [432-435, 437, 438]. The cells responsible for the slower decay of viral RNA in this second phase are not well understood, though it is hypothesized that they are either infected macrophages or infected resting CD4+ T cells [390, 434, 437-439]. These cells are estimated to decay with a half-life of approximately 14 days [390, 432, 434]. Following the two phases of HIV

RNA decay after ART initiation, an undetectable viral load (< 20-50 copies/mL) is generally achieved [422].

ART not only helps to clear actively-infected cells, it also allows for immunological rebound following a decrease in viral load. This can reverse much of the damage done to the CD4+ T cell compartment that was caused over the course of untreated HIV infection [55, 440, 441]. Like what is observed in viral RNA decay when starting ART, the rebound of CD4+ T cells upon ART initiation also appears to be biphasic [49, 55]. The increase in CD4+ T cells is initially quite rapid, followed by a slower period of gain associated with thymic output and the expansion of naïve and memory T cells [49, 55].

While ART typically leads to a rebound of CD4+ T cells and a significant decrease of HIV RNA, it also induces the decay of HIV DNA, as well. Upon initiation of ART, there is a rapid decline of CD4+ T cells that harbor HIV proviruses within the first year of treatment [40, 109, 393, 442]. Afterwards, between one and four years of ART, cells harboring HIV provirus decay at a slower rate [40, 109, 121, 393, 442]. After four years of ART, however, the number of these cells appears to plateau with limited decay thereafter, and sometimes even an increase is observed [49, 121, 393, 395, 442]. Thus, while ART can bring HIV viral loads down to clinically undetectable levels, it cannot clear all HIV genetic material that accumulates during infection.

1.7 The HIV-1 Reservoir

While ART can clear infected cells and promote the repopulation of healthy ones, it is not a cure for HIV. Due to the integration of the proviral genome into the genome of its host, a reservoir of cells harboring latent HIV provirus persists during long-term ART, requiring its lifelong administration. This is because in nearly every case, the cessation of ART ultimately

results in viral rebound as a result of the HIV reservoir [9, 10, 23]. Thus, the HIV latent reservoir remains a significant public health problem that still needs to be addressed.

Oftentimes the term "reservoir" is reserved for cells harboring intact, replication competent provirus and not those with a defective provirus. For the purposes of this text, cells with an intact HIV provirus will be called the "intact reservoir" and cells with a defective HIV provirus will be referred to as the "defective reservoir," recognizing the fact that this terminology is not the current standard in the field. When referencing simply the "reservoir," this refers to the compilation of all cells possessing a HIV provirus, regardless of whether it is intact or defective.

When HIV infects a host cell it can result in either an active infection, where the virus replicates inside the cell and ultimately causes it to die in the process, or a latent infection where the virus remains transcriptionally silent following provirus integration [9, 10, 443, 444]. When a person begins ART, actively infected cells and plasma viremia are rapidly cleared. This also prevents uninfected cells from becoming newly infected [432, 436, 439, 445]. However, ART cannot directly act on latently infected cells because the mechanisms of ART are directed at active stages of the HIV viral life cycle. Therefore, though ART can allow for a person living with HIV to live a near-normal lifespan [1], it does not represent a cure for HIV because at any given time, a small number of dormant intact reservoir cells can stochastically reactive and result in virus production [11-13, 18, 446]. In the presence of ART, the virus that is produced is unable to spread to other cells and the reactivated cell is killed either via viral cytopathic effects or immune-mediated clearance [134, 392]. If ART is discontinued, however, this stochastic reactivation results in a rebound of viremia [2, 9, 10, 23]. Therefore, in order to develop a cure for HIV, the elimination of the HIV reservoir will be necessary.

Several studies have shown that the majority of archived proviral sequences that persist on ART are genetically similar to those of circulating viruses near the time of ART initiation [24-26]. These findings suggest that the majority of cells that make up the HIV reservoir are established near the time of starting ART. The timing of ART initiation appears to impact the nature of the persistent reservoir during ART, as well, with differences observed in its dynamics between people who begin ART during acute infection compared to those who start during the chronic phase. In addition, there is data to suggest that the reservoir's natural rate of decay during continuous ART is faster for those who begin treatment during acute infection versus chronic infection [109, 121, 122, 447].

Perhaps most consistently, the size of the HIV reservoir is directly associated with the amount of time between seroconversion and ART initiation [86, 109-113, 115-120]. This is important because the size of the HIV reservoir tends to be inversely associated with the time to viral rebound upon ART interruption [117, 130, 448-451], suggesting that virologic control in the absence of ART may be possible if the size of the HIV reservoir is small enough. This concept received considerable attention with the "Mississippi baby" who was born to a mother living with HIV and received ART 30 hours after birth. This infant discontinued ART at 18 months of age and demonstrated undetectable viral RNA or DNA for 12 months, which at the time appeared to signify this infant may have potentially been cured as a result of very early ART initiation. At 30 months of age, however, this infant exhibited viral rebound, suggesting that even an exceedingly small reservoir is still capable of preventing an HIV cure [87].

While more studies are needed to test the hypothesis that an extremely small reservoir may result in a lack of viral rebound upon ART cessation, even if this is not the case it is still important to understand factors associated with a reduced reservoir size. This is because the size

of the reservoir directly correlates with levels of chronic immune activation that are maintained on ART [112, 115, 452, 453] which can lead to an increased risk of co-morbidities [212, 454-460], underscoring the importance of initiating ART as soon as possible after seroconversion [461]. Together, these findings suggest that factors near the time when a person begins continuous ART may be important for understanding what influences the dynamics of the reservoir that persists on treatment. Therefore, further work to identify other factors that correlate with reservoir composition near this time could prove important for identifying ways to reduce it.

Understanding the cell types that makeup the overall reservoir will be important, too, for informing cure strategies. Several studies have demonstrated that the HIV reservoir primarily consists of resting central memory CD4+ T cells [462-465], though there is accumulating evidence that naïve CD4+ T cells can also significantly contribute to its composition [466, 467]. Additionally, hematopoietic stem and progenitor cells (HSPCs) [468, 469], macrophages, and microglia also contribute to the pool of cells harboring HIV provirus on ART [204, 352, 353, 470]. Reservoir cells can contain either a defective or intact provirus, and a subset of the latter may encode for replication competent virus. The vast majority of proviral sequences that persist on ART, though, are defective, typically possessing deleterious mutations in *gag* and/or *pol* [17, 18, 38]. Across all cells harboring persistent proviral sequences within a person on ART, on average approximately 93-97% are defective [17, 18, 38]. Although these defective proviruses cannot produce replication competent virus, they still pose a problem for people living with HIV on ART. This is because viral proteins that are expressed on cell surfaces due to the transcription of defective HIV provirus are still recognized by the immune system and thus may contribute to the chronic immune activation that persists even during undetectable viremia on continuous ART [20, 389, 391, 453, 471].

Among the resting CD4+ T cells that make up the bulk of the reservoir, up to 99% of them typically demonstrate a memory phenotype. This is an important distinction because memory T cells tend to be long-lived which may contribute to the persistent nature of the HIV reservoir [464, 465, 472-475]. Notably, the infection of an effector CD4+ T cell in the process of transitioning to a memory CD4+ T cell allows for the viral life cycle to proceed up to integration, upon which viral transcription is downregulated and suppressed which may provide evidence for how and why some cells transition to a latent state upon HIV infection whereas most do not [476]. This also highlights the fact that because HIV proviral DNA is integrated into its host cell's genome, the factors that regulate cellular transcription of its own DNA also regulate transcription of viral DNA, thus influencing the maintenance of a latent state of infection [476-481].

Some of these factors include histone modifications such as the positioning of the proviral 5' LTR in a nucleosome downstream of a transcription start site which pauses RNA polymerase II and represses cellular transcription machinery, preventing transcriptional elongation of HIV genes [481, 482]. Histone post-translational modifications such as the binding of histone deacetylase-1 (HDAC1) at NF-kB binding sites in the proviral 5' LTR also reduces viral gene expression, further promoting latency [479]. These mechanistic examples represent but a few of the known cellular epigenetic modifications that promote viral latency [477, 478]. Regardless of the mechanism, however, the maintenance of HIV latency is dependent on the transcription of HIV *tat*, with reduced viral transcription resulting in Tat levels below what is necessary for productive HIV transcription/transcription elongation [483, 484].

Because HIV integrates its genome into the genome of its host cell which can then undergo cell division, this results in the presence of an identical provirus in identical integration sites in all of its daughter cells. Therefore, the HIV reservoir is largely made up of several clonal

populations of CD4+ T cells that harbor the same provirus sequence in the same chromosomal integration site [394, 485, 486]. Considering the high mutation rate of HIV, in addition to the variance of its provirus integration site, the probability of two cells having the exact same provirus sequence in the exact same integration site without being from the same clonal lineage is negligible [394, 485, 486]. Therefore, clonal populations can be identified via provirus and integration site sequence data [394]. The proportion of reservoir cells, as a whole, that is made up of any one specific clonal family continuously fluctuates as certain clonal families proliferate while others contract [396]. In total, it is estimated that over 50% of the cells that make up the latent reservoir are derived from the expansion of clonal families [487-489].

The proliferation of clonal families within the reservoir is influenced by several factors. For all T cells, clonal expansion is maintained by homeostatic proliferation, typically via IL-7. This indiscriminate promotion to proliferate for all T cells contributes to the genetic stability of the reservoir [490]. When T cells of a particular clonal population encounter their cognate antigen, however, this promotes the preferential proliferation and survival of specific clonal families [491]. In doing so, this provides a mechanism of long-term persistence of the reservoir and impacts the overall genetic evolution and composition of the reservoir over time. The integration site of HIV in its host cell's genome can also influence the proliferation of certain clonal families, with integration into certain genes such as MKL2 or BACH2 that promote clonal expansion promoting the persistence of clonal families that are integrated into such genomic sites [485, 489]. The expansion of clonal families has been suggested as a potential reason to explain the increase in reservoir size that is occasionally observed during the plateau of reservoir decay after several years of ART, counteracting the loss of other reservoir cells due to their natural decay during ART [49, 121, 393, 395, 442]. Furthermore, an inverse association exists between the

size of a proviral clonal family and both its ability to reactivate and the probability that the provirus is replication competent [465]. This suggests that cells with intact provirus may be preferentially cleared by the immune system [39, 40, 390, 465].

Our understanding of these reservoir dynamics come from a variety of assays used to characterize the HIV reservoir that persists on ART. One such method that has been used extensively to quantify the HIV reservoir, and has contributed significantly to our understanding of it, is the quantitative viral outgrowth assay (QVOA) [8, 22, 443, 492, 493]. The QVOA is an assay that involves culturing at limiting dilution CD4+ T cells isolated from a person living with HIV who is virally suppressed on ART. Using a single round of T cell activation, the cultured T cells are stimulated so that if they harbor an intact HIV provirus, they should in theory produce replication competent virus. Uninfected CD4+ T cells from a seronegative donor are added after stimulation which results in further virus production that can then be measured via qPCR or p24 ELISA. While this assay represented the gold standard for reservoir quantification for many years, it has several drawbacks including its time consuming and resource intensive nature, and its inherent underestimation of the reservoir because not every cell with an intact provirus will reactivate in the assay [18, 494].

The genetic characterization of the HIV reservoir can be performed as well to aid in understanding its composition via assays such as the full-length individual proviral sequencing (FLIPS) assay [394]. This assay uses specific PCR primers to amplify and sequence near full-length proviral genomes from isolated CD4+ T cell genomic DNA. Because the FLIPS assay amplifies a near full-length provirus, it can identify whether a viral genome is intact or defective via sequence analysis. An individual provirus is inferred to be intact by the lack of identification of any large indels, hypermutations, frameshifts, deleterious stop codons, inversion sequences, or

mutations in the 5' packaging signal [387]. One downside to FLIPS is its inability to adequately measure the size of the reservoir as it has been shown to be biased towards detecting proviruses with large internal deletions due to common first-round PCR failures in reactions involving longer, intact sequences. Additionally, the assay is significantly labor-intensive and costly which, together, makes the assay not well-suited for high-throughput studies [387, 394, 495].

A newer assay that can generate quantitative data about the HIV reservoir is the intact proviral DNA assay (IPDA) [38]. The IPDA is a two-target digital droplet PCR assay with primer probes binding to the packaging site and the RRE within the viral genome. Though it only probes two regions of the viral genome, sequence analysis has demonstrated that deletions or deleterious hypermutations found in these two highly conserved sites are found in approximately 97% of defective proviruses, allowing for a relatively accurate estimate of defective proviral sequences [38]. There are three possible successful outcomes when performing the IPDA. The first is a reading of double positive droplets that are inferred to be from an intact provirus. The second features droplets only positive for the packaging site which are inferred to be defective due to 3' deletions or hypermutations. Droplets that are only positive for the RRE site are inferred to be defective due to 5' deletions or hypermutations [38]. These outcomes of the IPDA can therefore allow for an estimated quantification of both the number of defective and intact proviruses that persist within the HIV reservoir. A major benefit of the IPDA is its high-throughput nature and as such can be used on samples from large cohorts [39, 40, 390].

Another method of reservoir quantification that can distinguish between intact and defective reservoir dynamics is the Q4 PCR method [496]. This method involves a near full-length HIV PCR amplification of CD4+ T cell genomic DNA at limiting dilution isolated from virally suppressed people living with HIV. Amplified copies are then interrogated by qPCR using

PCR probes specific for four sites on the HIV genome: *gag*, *pol*, *env*, and the packaging signal. Based on the results from the four target qPCR, the subsequent sequencing of the amplified DNA allows for confirmation of its intact status in addition to the ability to analyze the viral sequence and understand its clonality [496].

The Q4 PCR method is similar to the IPDA in that it can distinguish between intact and defective proviruses using primer probes, however the Q4 PCR method has several disadvantages compared to the IPDA. First, the Q4 PCR is much more difficult to perform on a largescale due to the necessity of provirus amplification at limiting dilution. It additionally has shown to have difficulty in achieving consistently successful near-full length genomic amplifications [497, 498]. However, the ability to analyze the provirus sequences is a major advantage compared to the IPDA. Yet, in a head-to-head comparison of the two methods using the same samples, the Q4 PCR method significantly underreported the number of intact proviruses, suggesting the IPDA may be a preferable assay to use if your primary focus is on comparing quantifications of the intact and defective reservoirs [498].

Like other reservoir measurement assays, though, the IPDA comes with several limitations, itself. For example, the estimated number of intact provirus copies is likely an overestimate as deleterious mutations can be found outside the two binding sites. In fact, a mathematical model has estimated that the IPDA results in the misclassification of approximately 5% of the investigated provirus sequences. This model additionally estimated that if the number of primer probe sites evaluated was increased, so could the assay's specificity. Though, classically, this would result in a subsequent decrease in its sensitivity [497].

Furthermore, because it only provides a yes/no output for the presence of two conserved regions of the viral genome, the IPDA is unable to provide viral sequencing or integration site

data. Without viral sequencing data, the assay cannot accurately predict if an inferred intact provirus encodes for a functional, replication competent virus or not. It is important to note, as well, that the original assay is designed specifically for subtype B HIV [38], limiting its utility for reservoir analysis of people in regions with the highest prevalence of HIV [19, 499] (Figure 1.7). Recently, however, a similar assay, the cross-subtype intact proviral DNA assay (CS-IPDA), was developed.

The CS-IPDA allows for more reliable reservoir quantification estimates among populations from a variety of geographic regions as it uses degenerate primers to accommodate known sequence heterogeneity in the 3 primer/probe binding sites in *gag*, *pol*, and *env*, and has been shown to reliably interrogate provirus sequences across subtypes [37]. The primers in the assay were developed through the modification of primers originally designed for a subtype B-specific multiplex ddPCR assay meant for quantifying intact proviruses [500]. When these modified primers were tested for their ability to quantify HIV DNA in samples from short-term cultures with a previously determined quantity of proviruses, their ability to detect HIV DNA was improved for non-subtype B samples (subtypes A, C, D, and CRF01_AE), with no decrease in detection of subtype B proviruses [37]. When the primers were used to quantify HIV DNA from J-Lat 5A8 cells which are known to contain one provirus per cell, using different known quantities of proviral copies the limit of detection of the CS-IPDA was determined to be one copy per one million cells [37]. Thus, the CS-IPDA can reliably quantify even low levels of HIV provirus across different HIV subtypes.

Although each of the listed methods have their own strengths and weaknesses, the application of these techniques and others have provided invaluable insight regarding the diversity, composition, and decay dynamics of the persistent HIV reservoir. Establishing the

typical baseline rate of reservoir decay, in addition to identifying factors that may modify it, is crucial for informing potential strategies to speed up this decay. In a landmark longitudinal study of 62 adults living with HIV who maintained viral suppression on ART for up to 7 years, the QVOA was used to determine the extremely stable nature of the persistent reservoir, demonstrating an estimated mean half-life of 44 months [501]. Extrapolating on this finding, this would mean that it would require roughly 73.4 years of continuous ART to completely eliminate the reservoir via natural decay on ART [501].

This calculation assumes that the reservoir decays at a constant rate over time, however. Later studies demonstrated that the reservoir decays in multiple phases, each with a different rate of decay [39, 40, 109, 121, 395, 442]. With more recently developed HIV reservoir characterization assays such as the IPDA, it is becoming increasingly clear that the long-term decay dynamics of the defective and intact reservoirs are significantly divergent, as well [39, 40, 390]. Emerging data has revealed that the intact reservoir appears to decay at a faster rate than the defective reservoir on long-term ART [39, 40]. Within the first three months of ART, the intact reservoir, as measured by IPDA, appears to decay with a half-life of approximately 13 days followed by a plateau in half-life of 19 months. This contrasts with the defective reservoir rate of decay following ART initiation which exhibits an apparent slow, monophasic decay at a rate that is considerably variable between individuals [390]. This area of study is still relatively new, with these findings having only been published within the past 3 years. Therefore, more studies characterizing the HIV reservoir that can differentiate between the intact and defective reservoirs can significantly contribute to our understanding of their differences.

Differences in reservoir dynamics have also been observed between children and adults living with HIV who are on ART. With the ability to detect vertical transmission of HIV for

newborn babies either *in utero* or postpartum in early life, infants who acquire HIV can be started on ART during very early infection [92, 502, 503]. Examples of this have demonstrated that very early ART initiation in children can lead to a lack of detectable immune response to HIV [504] and even incredibly limited, or altogether absent, detectable HIV DNA or RNA [92, 502, 503]. This has led to studies that have reported prolonged viral suppression in children after treatment interruption who started ART very early in life [81, 87].

Children who begin ART during chronic infection tend to have reservoirs comparable in size to those in adults [100, 505]. However, there are conflicting reports regarding the rate of reservoir decay during treated HIV infection in adults and children. Some studies suggest the HIV reservoir decays more slowly for children than adults during sustained ART [83, 506], whereas others have reported a faster decay among children [99, 447]. Others have suggested a similar rate of reservoir decay between adults and children [85, 507, 508]. An important difference between these studies is the age of the children in the cohorts, with some starting ART very early in life (< 6 months) [83, 99, 447, 509] whereas others began treatment at a later age [506, 507] which may make comparing the studies more difficult since the pediatric reservoir decays faster in children who start early ART compared to those who begin ART during chronic HIV infection [85, 99, 506]. The pediatric reservoir also tends to have a smaller percentage of intact proviruses compared to adults [92] which could potentially contribute to the differences observed in reservoir decay rates between these groups since the intact reservoir has been shown to be cleared more quickly than the defective reservoir [38-40, 390]. Additionally, cells in the pediatric reservoir have shown a greater resistance to reactivation than adult reservoir cells when stimulated *ex vivo* [91], suggesting possible differences in latency reversal kinetics in pediatric

reservoir cells which may also contribute to the differences in the rate of reservoir clearance in children and adults.

Despite apparent differences in reservoir dynamics between adults and children, studies agree that reservoir decay in the two groups occurs in similar phases, with the first year of ART representing the highest decay rate, followed by approximately one-to-four years of relatively modest decay, and a general plateau in decay after approximately four years of ART [85, 121, 442, 506, 507, 509]. Therefore, while the HIV reservoir does naturally decay during long-term ART in children, eventually this decay may become negligible which poses a problem for achieving the goal of inducing as small of a reservoir as possible. While early ART initiation is associated with the establishment of a smaller reservoir and increased reservoir decay in children [83, 85, 99, 447, 506], it is not always possible to begin a child on ART during acute HIV infection. Additionally, similar to what is observed in adults, the reservoir is maintained in children via clonal proliferation of certain reservoir cells, with evidence to suggest this may even occur as early as during fetal development [510, 511].

Furthermore, while several studies have been conducted on HIV reservoir dynamics among children who started ART soon after vertical transmission [79-83, 85-90, 92, 121], most of these studies have been conducted in resource rich settings like North America or Europe, thus predominantly focusing on subtype B [19]. However, the majority of children living with HIV live in sub-saharan Africa [34], where non-subtype B is predominant [19]. Additionally, far fewer studies exist regarding children who are living with HIV who began ART later in life, even though the majority of children who experience vertical HIV transmission initiate ART during chronic infection [512]. Thus, this represents a population whose reservoir dynamics have been severely understudied despite representing the majority of children living with HIV [512].

In summary, understanding the mechanisms behind the size and decay of reservoir cells during long-term ART, both in adults and in children, will be important for developing strategies to eliminate these cells. One area of research focus regarding factors that promote this natural decay has been on immune-mediated clearance of reservoir cells, either via CD8+ T cell killing [133, 134, 513] or antibody-mediated cytotoxicity [27, 30-32, 136]. Thus, understanding the adaptive immune response during HIV infection could offer clues for the development of novel HIV cure strategies.

1.8 Antibody Responses to HIV-1

Upon establishment of HIV infection, initial antibody responses to the virus develop within approximately one-to-two weeks in the form of either virion-IgM or virion-IgG antibody complexes [514-517]. This is soon followed by the appearance of freely circulating antibodies specific for the TM portion of Env (gp41) which typically arise between two-to-three weeks post-infection [176, 516-519]. These gp41-specific antibodies that are the first to arise may include antibodies that are cross-reactive with antigens present on bacterial gut flora and usually exhibit high levels of somatic hypermutation, which has led to the suggestion that they may be a recall of previously developed antibodies prior to HIV infection establishment [520-522]. This response is soon followed by the development of antibodies that target the Gag p24 antigen [517, 523]. Antibodies directed towards the SU portion of Env (gp120) appear around one-month post-infection, roughly two weeks after the first appearance of gp41-specific antibodies [517, 518, 523, 524].

The emergence of antibodies capable of neutralizing autologous virus typically do not develop until 3-12 months post-infection [319, 518, 525-527]. These neutralizing antibodies can

neutralize susceptible circulating viral strains, but loss of viral control inevitably occurs due to rapid viral escape from antibodies via a variety of evasion techniques [403, 528-534]. The virus takes advantage of the glycosylated nature of Env by adding and/or shifting glycosylation sites as a means of antibody escape via a "glycan shield" that can occlude neutralizing antibody access to its cognate epitopes [319, 526, 535-539]. Another mechanism of immune evasion by HIV is the induction of antibodies that target "decoy" epitopes. Neutralizing antibodies typically target functional Env trimers such as those located on the surface of replication competent virions [540-545] (Figure 1.4). When functional Env trimers are presented to the humoral immune system, this results in the development of antibodies specific for epitopes that are accessible when Env is in this conformation [545-548]. However, because gp120 and gp41 are bound noncovalently, the two subunits often disassemble which results in the development of antibodies specific for epitopes on gp120 monomers and gp41 stumps which are generally non-neutralizing as they do not target epitopes on functional Env trimers [545-547].

Additionally, because the Env trimer is conformationally flexible due to gp160 precursor cleavage, when gp120 binds to CD4 on a host cell a conformational change is induced in Env to an "open" state that reveals certain epitopes only accessible in this state [140, 541, 546, 549]. Therefore, neutralizing antibodies targeting these sporadically exposed epitopes do not interact with Env in its "closed" conformation which is the state Env is typically found in when on virion surfaces [546, 549, 550]. Aggregated gp160 released from the cytoplasm of dead infected cells can also result in the development of antibodies specific for epitopes found in this particular conformation [547, 548]. Antibodies targeting these decoy or occluded epitopes are typically non-neutralizing and dominate the overall Env-specific polyclonal antibody response, distracting

from proper development of an antibody response targeting the functional Env trimer and thus serving as another mechanism of viral immune escape [545-549].

As the continuous evolution of viral variants drives a corresponding evolution among the HIV-specific polyclonal antibody response, over time during chronic, untreated HIV infection the breadth and potency of the neutralizing antibody response generally increases and can expand to neutralize heterologous HIV viral variants representing viral strains not found within that individual [551-555]. For some people, their antibody response can even broaden to neutralize across HIV subtypes [93-96, 553, 556-561]. Among adults living with untreated HIV infection, a subset of approximately 15-30% develop a broadly neutralizing antibody response capable of targeting relatively conserved sites on Env, thus allowing for significant neutralization breadth across HIV variants [94-98, 552, 562, 563]. The development of a broadly neutralizing antibody response, however, does not confer improved prognostic outcomes for those who develop them [553, 558, 564, 565].

Several monoclonal broadly neutralizing antibodies (bNAbs) have been isolated and characterized from people with cross-subtype plasma neutralization breadth to understand the mechanism driving this breadth [559, 561, 566-568]. Some of the bNAbs that have been isolated to date have proven effective in preventing HIV infection via passive transfer in nonhuman primate (NHP) models suggesting their potential for HIV prevention strategies [569-573]. A recent clinical trial of passive transfer with a single monoclonal bNAb in a cohort of humans demonstrated limited prophylactic efficacy, however, with preventative capability dependent on circulating viral variants being susceptible to the specific administered bNAb [574]. The results of this clinical trial suggest that a cocktail of passively transferred bNAbs may be required for proper HIV prevention, similar to the necessity for a cocktail of drugs in ART [422]. Thus, both

the autologous antibody response developed during HIV infection and passive transfer of heterologous monoclonal antibodies have shown limited utility to impact HIV prevention and disease progression in studies of adults. Studies of antibodies in the context of pediatric HIV infection have reported similar findings, though perinatal HIV infection represents a unique antibody response to HIV including transmission in the presence of passively transferred maternal antibodies [575-580] and the more frequent development of bNAb responses [85, 87, 88, 90], suggesting a distinct immune environment for HIV infection in children.

1.9 Antibodies During Pediatric HIV-1 Infection

Pediatric HIV infection is unique compared to adult HIV infection for a variety of reasons. One unique aspect of perinatal HIV infection is that during the third trimester of gestation, maternal antibodies are transferred to the developing fetus from their mother which helps newborns fend off the onslaught of pathogens they will encounter upon entering the world while they develop the ability to properly mount their own immune responses [575-580]. When an infant is exposed to HIV via vertical transmission, they typically also concurrently have circulating HIV-specific maternal antibodies which may be capable of impacting HIV acquisition and/or pathogenesis [69, 70, 72, 575, 580-582]. Despite an immature immune system [267, 268], a potentially long duration of viral exposure *in utero* [166, 235, 237, 583], and likely repeated exposure events via breastfeeding [57, 255, 257, 584-586], when a mother living with HIV is not on ART, the risk of vertical transmission to their infant is approximately 20-30% (approximately 1% when a mother is on ART [587]) which is lower than what may be expected in the face of repeated exposures. Together, this suggests that passively transferred maternal antibodies may provide some protection from vertical HIV acquisition [69, 70, 72, 575, 580-582].

To understand if and/or how pre-existing maternal antibodies may impact HIV acquisition risk, studies have been conducted to understand whether maternal or infant antibody breadth is associated with a reduced risk of vertical HIV transmission. A number of early studies have, in fact, demonstrated a difference in heterologous plasma neutralization breadth between transmitting and non-transmitting mothers [588, 589]. A similar, more recent investigation that controlled for factors such as maternal viral load, CD4 count, and infant gestational age also demonstrated an association between maternal heterologous antibody breadth and a reduced vertical transmission risk [576, 590]. However, in other similar studies, no statistically significant difference in heterologous neutralization breadth was found between transmitting and non-transmitting mothers [67, 529]. Additionally, when *autologous* neutralization breadth was measured in a small group of transmitting and non-transmitting mothers, no statistically significant association was observed between antibody breadth and vertical transmission risk [591].

Although there is conflicting data to support the hypothesis that maternal antibodies may be protective in the setting of vertical HIV transmission, the aforementioned studies were solely focused on maternal neutralization breadth and not that of passively transferred antibodies circulating among exposed infants [67, 576, 588-591]. When similar studies that focused specifically on infants were performed, multiple reports demonstrated that the heterologous plasma neutralization breadth circulating among infants who vertically acquired HIV infection was not significantly different compared to those who did not [575, 582, 592, 593]. In one study, however, both infant and maternal plasma neutralization breadth were characterized against a panel of both heterologous viral variants and autologous viral variants found circulating within the mother of each infant-mother pair [592]. This study found that there was no significant

difference in the plasma neutralization breadth against neither heterologous nor autologous viral variants between infants who vertically acquired HIV compared to those who did not [592]. In contrast, non-transmitting mothers had a significantly higher heterologous, but not autologous, neutralization breadth compared to transmitting mothers [592].

Additional evidence to support the assertion that circulating maternal antibodies may impact vertical HIV transmission can be derived from studies that have cloned *env* variants from infant-mother pairs and directly tested their neutralization susceptibility to maternal plasma circulating near the time of transmission [594-599]. The results from across several different studies agree that viral variants circulating among infants soon after vertical transmission were significantly more resistant to neutralization by their matched maternal plasma compared to other representative viral variants circulating within their mothers [594-599]. These studies suggest that the T/F virus that is transmitted during vertical transmission could be an escape mutant that evolved to resist neutralization by maternal autologous antibodies.

Investigations into the possible mechanism of escape of vertically transmitted T/F viruses from maternal antibodies have shown frequent modifications to glycosylation sites present on Env [167, 594, 596, 597, 600]. Several vertically transmitted viral variants have been observed to have escaped from broadly neutralizing antibody responses circulating in the transmitting mother, with mutations in a common bNAb epitope target repeatedly found among vertically transmitted variants [596, 601]. The only two infant-derived monoclonal bNAbs isolated to date happen to both target this same common bNAb epitope [559, 602].

Whereas the influence of maternal neutralizing antibodies on vertical HIV transmission remains unclear, the impact of antibody-dependent cellular cytotoxicity (ADCC)-mediating antibodies in this context appears to be much more straightforward. ADCC occurs when an

antibody binds to its respective antigen with its Fab, or antigen binding, region. This allows for its Fc, or constant region, at the other end of the antibody to bind to CD16 on immune effector cells, forming a bridge between the antigen and the effector cell [603-606]. The effector cell then becomes activated and triggered to release inflammatory cytokines and chemokines capable of killing the cell to which it is attached via antibody binding [605, 606]. Although there are several cell types capable of participating in this process such as macrophages, monocytes, eosinophils, and neutrophils, the primary mediators of ADCC are Natural Killer (NK) cells [607].

Several studies analyzing the ADCC capacity of placentally-transferred antibodies among infants who both did, and did not, acquire vertically transmitted HIV across different cohorts have demonstrated a consistent finding that higher HIV Env-specific ADCC activity in infants indirectly correlates with vertical HIV acquisition risk [70, 72, 582]. Increased Env-specific ADCC activity in infants has also been consistently shown to be associated with improved survival outcomes [70, 72, 582, 608, 609]. There is, however, some discrepancy between studies examining the protective potential of ADCC-mediating antibodies in breast milk, with one study [581] reporting an inverse correlation between ADCC activity measured in breast milk and vertical transmission risk, and another similar study [70] failing to observe this association. Thus, ADCC may play an important role in the transmission and pathogenesis of pediatric HIV infection.

1.10 Antibody-Dependent Cellular Cytotoxicity in the Context of HIV-1 Infection

The impact ADCC-mediating antibodies can have on HIV infection dynamics is not limited to pediatric HIV infection. Some studies probing the mechanism of bNAbs in nonhuman primates (NHPs) [610, 611] and a humanized mouse model [612] have shown that when

deleterious mutations are introduced to the Fc region of a bNAb to prevent antibody-mediated immune effector functions, its ability to prevent HIV acquisition is also diminished. This suggests that antibody functions other than neutralization, such as effector functions like ADCC, may be important for bNAbs to demonstrate their full effects.

ADCC has been associated with a variety of clinical benefits in both humans and animal models within the context of HIV infection such as a decreased likelihood of HIV acquisition [70, 72, 581, 610-612], improved survival outcomes [70, 72, 582, 613], and elite controller status [614, 615]. The ability of antibodies developed during acute HIV infection to mediate ADCC has also been shown to correlate with viral load setpoint during chronic infection [616-618] and gp120-specific ADCC activity has been reported to associate with the rate of disease progression [616, 619]. Additionally, ADCC was the main correlate of protection identified in the moderately successful HIV vaccine trial, RV144, despite the vaccine's lack of induction of broadly neutralizing antibodies [620]. ADCC has also been suggested to play a role in clearing cells with integrated provirus that persist on ART [27, 28, 30, 123, 136, 139, 142].

In order to understand the various ways ADCC-mediating antibodies can influence HIV infection, methods to measure their ADCC activity are necessary. There are several different methods capable of measuring antibody ADCC activity, though similar findings have been reported using different ADCC assays such as the association of higher Env-specific ADCC and decreased vertical transmission risk and pathogenesis [70, 72, 582]. Each assay has its own advantages and disadvantages in terms of its ability to properly assess physiologically relevant levels of ADCC activity [621]. Assays used to measure ADCC can generally be divided into two categories based on the target cells used: env-coated or infected cells [621].

In assays using env-coated target cells, primary CD4+ T cells or CEM.NKr cells [622] are coated with recombinant Env proteins such as gp120 [36, 72, 582] or with inactivated viral particles [285, 623]. Importantly, binding of recombinant viral proteins or inactivated virus to the target cell is mediated by interactions with CD4 on its surface [624]. Once the inactivated virus or viral proteins are bound to the target cell, antibodies and effector cells are added to facilitate ADCC. The ADCC-mediated killing of target cells can be quantified either through a ^{51}chromium release assay [625] or by loss of a viability dye by flow cytometry in a method termed the Rapid and Fluorometric ADCC assay (RFADCC) [36]. Measuring ADCC using the RFADCC assay has proven reliable, with ADCC data generated using this method having correlated with clinical outcomes in several studies [72, 581, 582] as well as with ADCC data generated using other ADCC measurement techniques [72].

One limitation of the RFADCC assay, however, is that measuring the loss of cellular dyes could potentially also measure antibody-dependent cellular phagocytosis or even trogocytosis, which is when part of a plasma membrane is sheared off from one cell by another [626]. Methods that employ env-coated target cells, in general, have an additional limitation that the viral proteins attach to the target cells by binding to CD4. In doing so, antibodies specific for the CD4 binding site on Env gp120 are occluded and therefore are not measured by these assays [624]. Additionally, coating with recombinant proteins can only measure ADCC activity of antibodies specific for linear epitopes or Env protein monomers, thus missing detection of ADCC mediated by antibodies that bind to conformational epitopes [627]. To work around these limitations, ADCC assays that employ infected target cells can be used, as they present conformational epitopes like what is seen in natural infection.

Similar to the env-coated target cell methods, ADCC assays using infected cells can use either primary CD4+ T cells or a CEM.NKr cell line. To measure ADCC, target cells are infected with an infectious HIV molecular clone (IMC), with infected cells identified by p24 staining. ADCC-mediated killing is therefore measured by calculating the percentage loss of p24+ cells in a population via flow cytometry [628]. Similarly, instead of identifying infected cells by p24 staining, IMCs that encode for reporter genes like luciferase or GFP can be used [137]. A limitation to using an IMC with a reporter gene, however, is that insertion of the reporter gene may alter the proper function of Nef which is a viral protein important for ADCC evasion [140]. However, using a cell line that encodes a Tat-driven luciferase gene can allow for a similar method of identification of target cells without the potential for interfering with the viral Nef protein [629]. A significant limitation to using infected cells as target cells in an ADCC assay is the fact that Env trimers regularly shed gp120 monomers which can bind to the surface of nearby uninfected bystander cells, coating their surfaces similar to env-coated ADCC assays [326, 546]. This can then result in off-target measurements and skew the reported ADCC quantification.

Yet another method to measure ADCC is by measuring indicators of effector cell activities instead of directly measuring killing of target cells. Methods like the GranTox-iLux assay measure granzyme B activity which is released by effector cells during ADCC [630]. Other indicators of effector cell function that can be measured are factors like intracellular IFN-γ [631] or cell surface expression of CD107a [632]. As noted, these methods do not directly measure ADCC, but serve as a proxy for ADCC as they measure effector cell activities. From these various assays, several studies have reported the impact ADCC-mediating antibodies can have on HIV transmission [70, 72, 581, 610-612], pathogenesis [70, 72, 582, 613], and even cells of the HIV reservoir [27, 28, 30, 123, 136, 139, 142].

1.11 Studies Regarding the Impact of HIV-1-Specific Antibodies on the HIV-1 Reservoir

The ability to clear cells of the HIV reservoir via immune effector functions has received considerable attention. This is because enhanced cytotoxic T lymphocyte (CTL) function has been identified as a hallmark of elite controllers who are people who can maintain undetectable viral loads in the absence of ART [134, 513, 633]. Importantly, elite controllers have also been shown to have lower levels of HIV proviral DNA compared to progressors [634, 635], and CTLs from elite controllers have demonstrated an ability to recognize and kill CD4+ T cells that contain HIV provirus in an *in vitro* model [134]. Like CTLs, NK cells can also recognize and kill infected target cells [136]. In an infected cell, HIV Env buds from the plasma membrane which results in its exposure on the cell's surface where it can be recognized and labeled by Env-specific antibodies [288]. Importantly, although the antibodies found in the sera of people living with HIV predominantly target epitopes on Env that are exposed in the Env trimer's "open" state which are typically non-neutralizing [141, 541, 550], many of these antibodies can often still facilitate ADCC [141, 550].

HIV can evade ADCC mediated by these antibodies, though, by limiting Env conformational changes into an "open" state via its accessory proteins Vpu and Nef. It does so by downmodulating CD4 in infected host cells, thus limiting CD4-Env interactions on the infected cell surface [141, 340]. To further avoid antibody recognition of Env in this transient state, by limiting Env-CD4 interactions, Vpu and Nef also limit the amount of Env trimer on a cell's surface which may diminish antibody clustering, preventing ADCC from killing the infected cell [6, 140, 141, 288, 340]. In addition, Vpu and Nef also downmodulate co-receptors important for NK cell activation, further avoiding ADCC-mediated killing of its host cell [3, 636, 637].

Therefore, in theory, ADCC may have the capacity to recognize and clear reactivated reservoir cells, but HIV immune evasion mechanisms may ultimately prevent this from occurring.

There is evidence from animal model studies, however, that suggest ADCC-mediated clearance of HIV reservoir cells is in fact possible. In a study of nonhuman primates living with chronic SHIV infection, the administration of a monoclonal bNAb resulted in not only a decrease of plasma viremia via its neutralizing ability, but of proviral DNA in the peripheral blood, gastrointestinal mucosa, and lymph nodes as well via its capacity to facilitate antibody-mediated effector functions. Some of the animals in the study even maintained long-term virological suppression despite only receiving one bNAb infusion [638]. In another study using a humanized mouse model involving mice injected with PBMCs isolated from people living with HIV who were long-term virally suppressed, in the absence of ART these mice ultimately developed rebound viremia. When a monoclonal bNAb was administered to the mice, however, there was a significant delay in viral rebound compared to mice who received a FcR^{Null} version of the same bNAb which knocked out its ability to facilitate antibody-mediated effector functions [29]. These results suggest that replication competent reservoir cells may have been cleared via Fc-mediated effector functions such as ADCC in this study.

In yet another humanized mouse model study that used mice with functionally active NK cells, PBMCs isolated from people living with HIV who were virally suppressed were injected into mice and treated either with autologous plasma from the PBMC donors plus a CD4 mimetic compound, or just a CD4 mimetic compound alone [142]. The addition of a CD4 mimetic compound that can bind to Env induces its "open" state, mimicking the change in Env conformation that occurs when it binds to CD4 on a host cell [137, 142]. Comparing the two groups, viral rebound occurred in the mice treated solely with the CD4 mimetic compound,

whereas viremia was not detectable in five of six mice treated with both the CD4 mimetic compound and autologous donor plasma within the same timeframe [142]. The data from this study therefore suggests that circulating antibodies specific for epitopes that are accessible when Env is in its "open" state may eliminate autologous, replication competent reservoir cells *in vivo*.

While animal studies typically offer a more streamlined approach for hypothesis testing compared to similar studies performed in humans, they do not always perfectly mirror human physiological mechanisms. We can glean some information about the ways antibodies may impact the HIV reservoir in humans, however, from various clinical trials that administered bNAbs to people living with HIV. Across various studies, bNAb administration consistently reduced viremia in people living with HIV, with consistent viral rebound after waning of the administered bNAbs [392, 639-644]. Relatively few of these clinical trials measured the impact the antibody treatments had on the HIV reservoir, though those that did demonstrated conflicting results [392, 642]. In one study, six people living with HIV who were virally suppressed on ART received two infusions of a monoclonal bNAb which did not result in a measurable difference in reservoir size before and after the bNAb treatment [642].

In contrast, another clinical trial administered seven doses of a combination of two different monoclonal bNAbs over 20 weeks to people living with HIV who continued their ART regimen during the trial [392]. For defective proviruses, there was no measured difference in their absolute number between start and finish of the trial. There was, however, a moderate but significant reduction in the number of intact proviruses following the seven bNAb infusions [392]. Together, studies in both animal models and human clinical trials promote the possibility that antibodies can be used to eliminate cells in the HIV reservoir, likely in a Fc-dependent

manner. Studies analyzing the ability of autologous antibodies to mediate reservoir clearance in humans are largely lacking, however.

1.12 Dissertation overview

The HIV pandemic has resulted in a tremendous loss of human life [34] since it was first reported over four decades ago [48], and although today ART can allow for a person living with HIV to live a near-normal lifespan [1], there continues to be no cure due to a reservoir of latently infected cells that persists on ART [2-13]. In addition to adults, children represent a significant number of people living with HIV and continue to acquire HIV through vertical transmission despite effective prevention strategies [149-151]. While there is a large body of literature focused on HIV reservoir dynamics in adults, with the goal of informing strategies for reservoir elimination, far fewer studies have been done regarding reservoir dynamics in children.

Additionally, whether in adults or children, there is a general dearth of studies investigating the ways autologous antibodies can impact the HIV reservoir despite immune-mediated clearance of the reservoir being a major focus of cure strategies [129]. Thus, the goal of the work described in this thesis is to improve our understanding of ways autologous antibody responses during chronic HIV infection can impact aspects of the HIV reservoir such as size and composition. Using samples from children living with chronic HIV infection both at the time of ART initiation and after prolonged ART adherence, the work described here tested the hypothesis that the ability of circulating antibodies present at ART initiation to mediate ADCC against HIV Env inversely correlates with the size of the HIV reservoir that is established after ART is begun. The results of this study will hopefully contribute to informing novel strategies to eliminate the persistent HIV reservoir that can finally work towards an HIV cure.

Chapter 2. Higher HIV-1 Env gp120-Specific Antibody-Dependent Cellular Cytotoxicity (ADCC) Activity is Associated with Lower Levels of Defective HIV-1 Provirus

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Author Contributions:

This work was made possible as the result of a collaborative effort by a brilliant team of scientists. Elizabeth Malecho-Obimbo, Grace John-Stewart, Dalton Wamalwa, and Julie Overbaugh were involved in the establishment, management, and execution of the original study from which the samples used in this study originated, including clinical data sample acquisition. Ryan Yucha, Grace John-Stewart, Julie Overbaugh, and Dara Lehman conceived of the study. Ryan Yucha and Zak Yaffe designed, performed, and analyzed the ADCC experiments. Morgan Litchford and Carolyn Fish designed, performed, and analyzed the CS-IDPA experiments. Julie Overbaugh and Dara Lehman were involved in the design and analysis of both types of experiments. Ryan Yucha, Morgan Litchford, Carolyn Fish, Zak Yaffe, Barbra Richardson, Julie Overbaugh, and Dara Lehman worked together on the data analysis involved in the study. Grace John-Stewart, Julie Overbaugh, and Dara Lehman were involved in funding acquisition. Ryan Yucha was the primary organizer of the manuscript writing, with contributions from all other authors towards the manuscript.

2.1 Abstract

A cure for HIV-1 (HIV) remains unrealized due to a reservoir of latently infected cells that persist during antiretroviral therapy (ART), with reservoir size associated with adverse health outcomes and inversely with time to viral rebound upon ART cessation. Once established during ART, the HIV reservoir decays minimally over time, thus understanding factors that impact the size of the HIV reservoir near its establishment is key to improving the health of people living with HIV and for the development of novel cure strategies. Yet, to date, few correlates of HIV reservoir size have been identified, particularly in pediatric populations. Here, we employed a cross-subtype intact proviral DNA assay (CS-IPDA) to quantify HIV provirus between one- and two-years post-ART initiation in a cohort of Kenyan children (n = 72), which had a median of 99 intact (range: 0 – 2469), 1340 defective (range: 172 – 3.84 x 10^4), and 1729 total (range: 178 – 5.11 x 10^4) HIV proviral copies per one million T cells. Additionally, pre-ART plasma was tested for HIV Env-specific antibody-dependent cellular cytotoxicity (ADCC) activity. We found that pre-ART gp120-specific ADCC activity inversely correlated with defective provirus levels (n = 68, r = -0.285, p = 0.0214), but not the intact reservoir (n = 68, r = -0.0321, p-value = 0.800). Pre-ART gp41-specific ADCC did not significantly correlate with either proviral population (n = 68; intact: r = -0.0512, p-value = 0.686; defective: r = -0.109, p-value = 0.389). This suggests specific host immune factors prior to ART initiation can impact proviruses that persist during ART.

2.2 Introduction

In its fourth decade of reported cases [48], the ongoing HIV-1 (HIV) pandemic has resulted in roughly 40 million people currently living with HIV, with a disproportionate amount of the global burden located in low and middle- income countries primarily in sub-Saharan Africa [34, 645]. Though access to antiretroviral therapy (ART) can allow for a near-normal lifespan [1], a cure for HIV remains elusive as the virus can persist during ART as a latent provirus due to the integration of the HIV genome into that of its host cell. This results in the formation of a reservoir of long-lived, quiescent cells harboring latent HIV provirus capable of host immune evasion and stochastic reactivation, all of which represent a barrier to cure [2, 8-10]. While the vast majority of persistent provirus during ART is defective [18, 20, 646] due to large internal deletions or hypermutations [15, 16], a fraction of proviruses are intact, of which a subset is able to produce replication competent virus. As a result, cessation of ART ultimately results in rebound of viremia, necessitating life-long administration of ART [2, 23] [9, 21, 647].

Despite viral suppression on ART, people living with HIV tend to have increased immune activation and inflammation markers [648-650] which is associated with a litany of adverse health outcomes such as cardiovascular disease [651, 652], neurological disorders [653], diabetes [654], cancer [655, 656], and accelerated aging [212, 657-659]. Cells harboring HIV provirus, whether intact or defective [20, 389, 391, 660-662], are drivers of this chronic immune activation due to their ability to persistently produce and present viral antigens [663], with the size of the reservoir positively associated with inflammation markers [664, 665] and risk of comorbidities [653]. Additionally, several studies have demonstrated an inverse relationship between HIV reservoir size and time to viral rebound upon ART interruption, fueling speculation that a functional cure for HIV may be possible by limiting the reservoir's size [117, 130, 451, 666].

Though the size of the reservoir that persists on ART can vary significantly between people, its rate of decay is low across individuals [109, 501]. Thus, it is important to identify correlates of a reduced HIV reservoir and to develop interventions to mediate such a reduction [667].

The notion that an exceedingly low reservoir size can result in a functional cure has received considerable attention in the setting of pediatric HIV infection. There have been several reported cases of children with extremely small reservoirs who initiated ART during very early life who maintained viral suppression for years after ART interruption, perhaps the most notable being the "Mississippi baby" [81, 87, 668, 669]. Larger studies corroborate these findings, revealing that ART initiation within the first year of life is beneficial for limiting pediatric reservoir size [87, 92, 670]. While early ART initiation in children is common in high resource settings like Europe and North America, children in sub-Saharan Africa who represent the majority of children living with HIV typically begin ART during chronic HIV infection [512]. Initiation of ART during chronic infection in children results in a latent reservoir size comparable to that observed in adult populations [100], with long-term ART in children also associated with adverse health outcomes and increased immune activation [671-673]. Yet studies of the pediatric HIV reservoir, especially those related to its establishment during chronic HIV infection, remain limited compared to those in adults. Additionally, despite representing a smaller fraction of global HIV cases, most studies on this subject are conducted in resource-rich countries where HIV subtype B is dominant [674, 675]. Thus further work to understand factors that influence the HIV reservoir in pediatric cohorts, particularly in cohorts representing populations where HIV prevalence is highest and the dominant circulating strains are non-subtype B, is crucial [499, 676, 677].

Although HIV integrates its genome into host cells during viral replication throughout untreated infection, the majority of archived proviral sequences that persist during ART appear to be seeded near the time of ART initiation as they are typically genetically similar to those of circulating viruses at that time [25, 26, 678]. This suggests that factors present at ART initiation may impact reservoir seeding and composition, and that interventions targeting the HIV reservoir may be advantageous to initiate at this critical time. The host immune response has been implicated as one such factor. For example, studies have shown that cells harboring latent provirus exhibit resistance to CD8+ T cell killing suggesting selection for CTL escape [679-681], however CD8+ T cell depletion in macaques at ART initiation does not impact the size of the established SIV reservoir [682]. Conversely, broadly neutralizing antibodies (bNAbs) have been shown to prolong viral suppression after ART interruption in humanized mice [683, 684], nonhuman primates [638, 685], and humans [32, 392, 643, 644, 686]. This suggests that in addition to neutralizing capabilities, these administered antibodies may also reduce the latent reservoir. Indeed, animal models have demonstrated the ability of bNAbs to clear HIV-infected cells [32] and even interfere with reservoir establishment through Fc-FcR-mediated mechanisms [683] implying that Fc-mediated effector functions, including antibody-dependent cellular cytotoxicity (ADCC), could influence reservoir establishment.

While these studies focus on the effect of heterologous antibody therapies, fewer studies have investigated the ability of antibodies induced by natural infection to impact the HIV reservoir. A recent study showed that contemporaneous antibodies blocked the viral outgrowth of a majority of viruses in the latent reservoir [687], indicating the ability of infection-induced antibodies to recognize and inhibit reactivated virus. Additionally, ex vivo studies have demonstrated the ability of autologous antibodies to mediate ADCC against paired reactivated

CD4+ T cells [28, 30]. Together, these studies suggest autologous antibodies may influence the HIV reservoir through mechanisms such as ADCC. HIV Env-specific ADCC activity has previously been shown to correlate with clinical outcomes including HIV transmission/acquisition [70, 581, 620, 688], survival [70, 72, 582, 608, 689], and elite controller status [614, 615, 690, 691]. However, no studies to date have evaluated the association between HIV infection-induced ADCC and proviral DNA levels that persist during ART.

In this study, we tested the hypothesis that HIV Env-specific ADCC activity at the time of ART initiation inversely correlates with the size of the established HIV reservoir. We leveraged samples from the Pediatric Adherence Diary [692] study [693] that enrolled ART-naïve children in Kenya living with HIV, with longitudinal samples collected over several years during continuous ART. Using the Rapid and Fluorometric ADCC (RFADCC) assay [36] that was previously shown to correlate with pediatric clinical outcomes [72, 582], and the newly developed Cross-Subtype Intact Proviral DNA Assay (CS-IPDA) [37], this study investigated the relationship between pre-ART ADCC activity and levels of HIV provirus in children during ART. Our findings suggest that higher HIV Env gp120-specific ADCC activity in plasma at the time of ART initiation may reduce total and defective provirus levels during ART, but not the intact reservoir.

2.3 Results

2.3.1 Study Population and Baseline Characteristics

Children living with HIV in Nairobi, Kenya were provided ART at enrollment into the Pediatric Adherence Diary [692] study [693] and monitored through two years of longitudinal follow-up visits. For this study, plasma and PBMC samples that met our inclusion criteria, described in methods, were available from 72 of the participating children. The median age at

study enrollment was 4.92 years, ranging from 1.29 to 12.7 years. Pre-ART median viral load in this cohort was 5.96 $\log_{10}$ copies/mL (min: 4.18, max: 6.96), with median CD4 percent at 6.30% (min: 0.700%, max: 73.4%) and a median CD4 count of 354 cells/mm3 (min: 15.0, max: 2009). Both CD4 % and CD4 count are reported because in newborns, the absolute number of T cells is much higher than in adults and gradually decreases to adult-like levels between the ages of six and 12, thus CD4 % is used when comparing children of varying ages [694, 695]. Of the children in our study with CD4 % data at study enrollment, 79% (n = 45) had a CD4 % <15% which is considered immunosuppressed, and 21% (n = 12) were not immunosuppressed (CD4 >15%). Participants assigned female at birth represented 54% (n = 39) of the cohort, with those assigned male representing 46% (n = 33). Following study enrollment, nearly all participants (n = 70) started an ART regimen consisting of one non-nucleoside reverse transcriptase inhibitor (NNRTI) and two different nucleoside reverse transcriptase inhibitors (NRTI), with one participant receiving a triple NRTI regimen and one receiving a combination of one NNRTI, one NRTI, and one protease inhibitor (Table 1).

N = 72	Median	[Min, Max]
Age at Enrollment		
Months	59	15.5, 152
Years	4.9	1.3, 12.7
Viral Load (log_{10} c/mL)	5.96	4.18, 6.96
CD4 %	6.3	0.70, 73.4
CD4 Count (cells/mm^3)	354	15, 2009
	N	%
Gender		
Male	33	46%
Female	39	54%
ART Regimen		
NNRTI, NRTI	70	97.2%
NRTI	1	1.4%
NNRTI, NRTI, Protease Inhibitor	1	1.4%

Table 2.1. Cohort Descriptive Statistics

Abbreviations: Min, minimum; Max, maximum; c/mL, copies/mL; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nuceloside reverse transcriptase inhibitor

2.3.2. Quantifying Persistent HIV Provirus

The CS-IPDA [37], a three-target digital droplet PCR, was used to measure the number of intact and total proviruses per one million CD4+ T cells using available cryopreserved PBMCs obtained between 12 and 24 months post-ART initiation during viral suppression. The number of defective proviruses, defined as those lacking at least one region of the genome detected by CS-IPDA, was determined by subtracting the number of intact from the total HIV proviral copies. Though this assay cannot directly measure replication competence and may overestimate reservoir size if defects in the genome occur outside of the probed regions, intact (containing all three targets) provirus is used as a proxy measure for the replication competent reservoir, as IPDA measurements correlate with results from the quantitative viral outgrowth assay (QVOA) [38]. Due to the QVOA's requirement for a high volume of sample input, the CS-IPDA was

employed as it is a high-throughput assay that requires less sample volume, can interrogate provirus across clades, and has an absolute limit of detection of one copy per reaction [37]. In this study, there was a considerable range in the number of proviral copies per one million CD4+ T cells measured for total (median: 1729, min: 178, max: 5.1 x 104), intact (median: 99, min: 0, max: 2469), and defective (median: 1340, min: 172, max: 3.84 x 104) provirus (Figure 1A). As has previously been observed [8-10], the vast majority of detected provirus was defective (mean % defective: 86.3%, range: 6.5 – 100%).

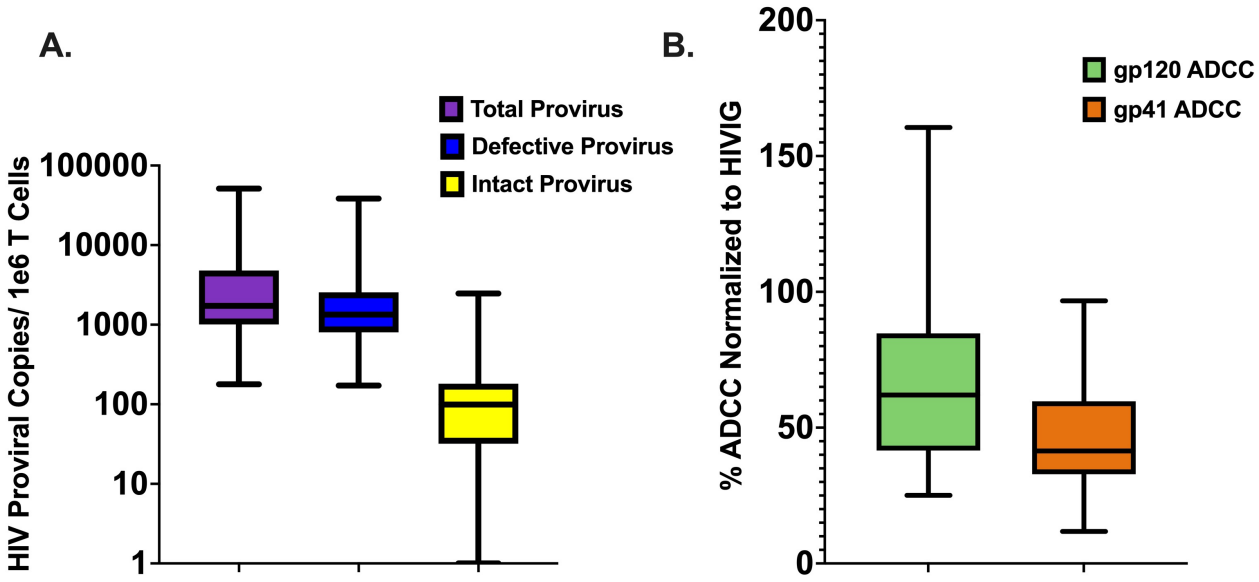


Figure 2.1 Distribution of HIV Proviral Copies and ADCC Activity Measured Across the Cohort.

(A) Distribution of the number of HIV proviral copies measured across the study cohort for total (left) (n = 72), defective (middle) (n = 68), and intact (right) (n = 68) provirus; **(B)** Distribution of ADCC activity measured across the study cohort for both gp120 (left) (n = 72) and gp41 (right) (n = 72).

2.3.3. HIV Env gp120-Specific ADCC Activity is Inversely Associated with Levels of Defective Provirus

Most of the HIV reservoir is established near the time of ART initiation [24-26], thus plasma ADCC activity against HIV Env gp120 was evaluated at study enrollment just prior to ART start. We independently tested two gp120 antigens, BG505 and BL035, which were both derived from Kenyan infants living with HIV. The average ADCC activity for each participant measured against BG505 was highly significantly correlated with ADCC activity measured against BL035 ($r = 0.773$, p-value = <0.0001) (Supplementary Figure 1A). The results for both antigens across three replicates were averaged for each child to report one gp120-specific percent ADCC. Median cohort ADCC activity against gp120 was 62.0%, ranging from 25.1% to 161% when normalized to the activity of a standard HIV plasma pool (Figure 2.1B).

To test our hypothesis that pre-ART HIV Env-specific ADCC activity is inversely associated with the size of the HIV reservoir, we first examined how pre-ART gp120-specific ADCC activity correlated with levels of total HIV provirus during ART. The results demonstrated a trend for an inverse association between HIV Env gp120-specific ADCC activity and the level of total provirus that persists during ART ($r = -0.214$, p-value = 0.0707) (Figure 2A). Since total HIV provirus comprises both intact and defective proviruses, we repeated the analysis for these two proviral categories separately. Based on studies demonstrating faster natural decay of cells harboring intact compared to defective provirus on long-term ART [39, 40, 390, 696], we anticipated an inverse association with gp120-specific ADCC activity and the size of the intact reservoir, but not with levels of defective provirus. To our surprise, there was no observed association between the size of the intact reservoir and gp120-specific ADCC activity ($r = -0.0321$, p-value = 0.800) (Figure 2.2B). In contrast, the results did demonstrate a

statistically significant, moderate inverse association between gp120-specific ADCC activity and levels of defective HIV provirus (r = -0.285, p-value = 0.0214) (Figure 2C). We also performed these same analyses with a more restrictive inclusion criteria to only include reservoir measurements from samples taken with viral suppression both at reservoir measure as well as for six months prior. This yielded similar results, demonstrating a trend for an inverse association between gp120-specific ADCC and levels of defective provirus (pearson correlations: total: r = -0.155, p-value = 0.208; intact: r = 0.0194, p-value = 0.881; defective: r = -0.219, p-value = 0.0867). In this analysis a decrease in statistical significance was observed, potentially due to a decrease in statistical power (total: n = 68 versus n = 72; intact and defective: n = 62 versus n = 65).

To further examine these associations, each participant was stratified based on ADCC activity relative to the median ADCC activity of the cohort. The two groups were labeled either "ADCC > Median" or "ADCC < Median." T-tests were performed to assess differences in mean HIV proviral copies between the two groups. This analysis did not demonstrate significantly fewer total HIV proviral copies, on average, in the ADCC > Median compared to the ADCC < Median group (p-value = 0.112) (Figure 2D). There was also no significant difference for the mean number of intact proviral copies between the two groups (p-value = 0.420) (Figure 2E). Additionally, the ADCC > Median group also did not demonstrate statistically significantly fewer defective proviral copies compared to the ADCC < Median group, though the results appeared to potentially be in that direction (p-value = 0.176) (Figure 2.2F). To further separate those with high and low ADCC, a binning approach was performed by including participants with ADCC activity at, or above, the cohort 75th percentile into an "ADCC High" group (n = 18) and participants with ADCC activity at, or below, the 25th percentile into an "ADCC Low"

group (n = 18). The results demonstrated no change in statistical significance for total provirus (p-value = 0.254) or intact provirus (p-value = 0.280), but did demonstrate a shift to a statistically significant difference between the two groups for mean defective proviral copies (p-value = 0.0232) despite the smaller sample size (Supplemental Figure 2.2A-C).

To address potential confounding effects, we ran univariate analyses to test for an association of age at ART start, pre-ART viral load, CD4 %, and CD4 count, independently, with the levels of each proviral category. While we do not know the exact timing of seroconversion for each child, we assume vertical transmission near the time of birth and use participant age at ART start as a proxy for time to ART initiation. In univariate analysis, none of the potentially confounding factors we analyzed were significantly associated with the levels of any proviral category in our cohort. However, as several studies have reported an association with time to ART initiation and the size of the HIV reservoir in children [668, 697, 698], we performed a multivariate linear regression controlling for time to ART initiation in the model. This analysis also demonstrated a moderate inverse correlation of gp120-specific ADCC activity with levels of defective HIV provirus in both a univariate linear regression (coefficient = -0.635, p = 0.0202) and a multivariate model controlling for age at ART start (coefficient = -0.588, p = 0.0414) (Supplementary Table 1). Taken together, the results suggest increased ADCC activity against HIV Env gp120 may be associated with a reduced number of defective, but not intact, copies of HIV provirus during ART.

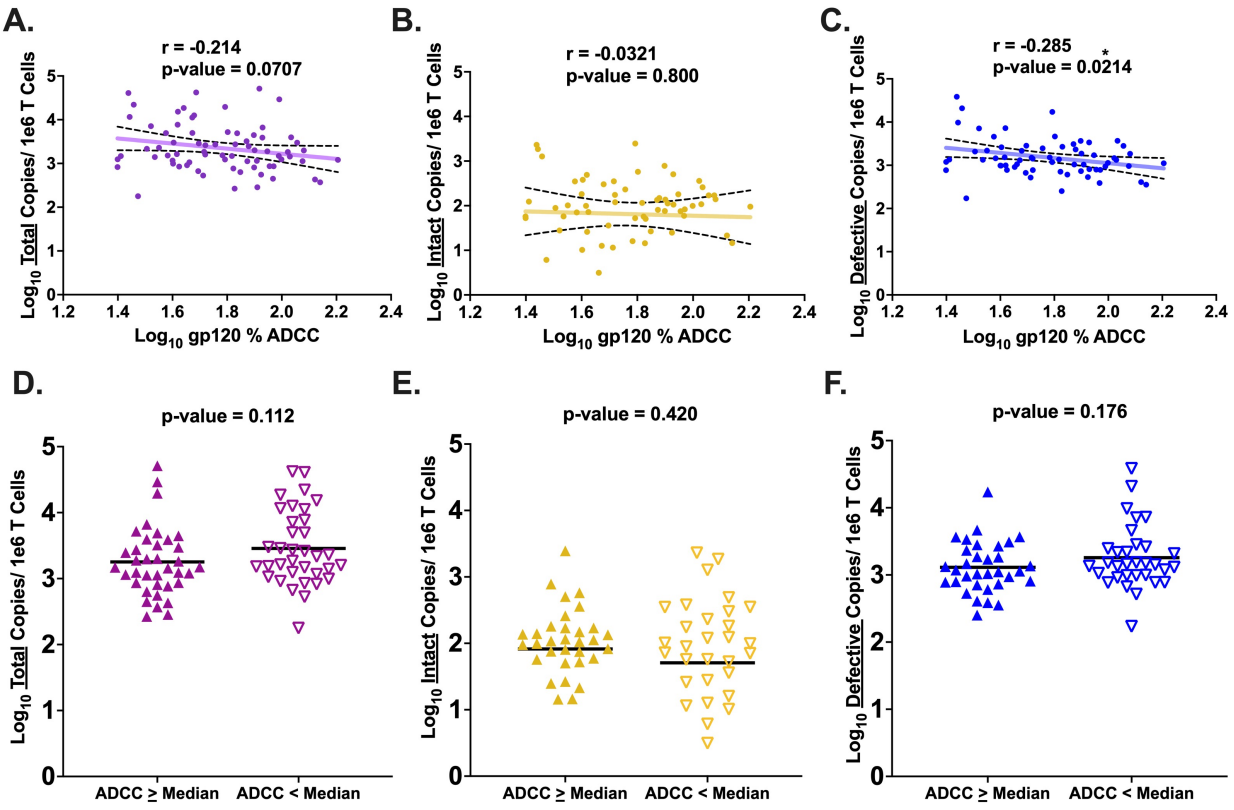


Figure 2.2 Association Between gp120-Specific ADCC Activity and Levels of HIV Provirus.

(A-C) Pearson correlation of $\log_{10}$ gp120-specific ADCC activity and copies of HIV provirus measured for each proviral category. The best fit line and its 95% confidence interval is imposed onto each graph. **(A)** Total HIV provirus (n = 72); **(B)** Intact HIV provirus (n = 68); **(C)** Defective HIV provirus (n = 68); **(D-F)** Unpaired t test comparing mean $\log_{10}$ HIV proviral copies between "ADCC > Median" and "ADCC < Median" groups. Participants were stratified into either group based on a participant's ADCC activity in relation to the cohort median; Participants at, or above, cohort median were labeled "ADCC > Median" with those below the cohort median labeled as "ADCC < Median." **(D)** Total HIV provirus (n = 72); **(E)** Intact HIV provirus (n = 68); **(F)** Defective HIV provirus (n = 68). *denotes a p-value < 0.05

2.3.4. HIV Env gp41-Specific ADCC Activity is Not Associated with Levels of Persistent HIV Provirus

Since HIV Env consists of both gp120 and gp41, we next wanted to determine if gp41-specific ADCC activity demonstrated the same associations with levels of HIV provirus as those observed for gp120-specific ADCC. Median gp41-specific ADCC activity across the cohort was 41.4%, ranging from 11.8% to 96.7% (Figure 2.1B). The ADCC activity specific for gp41 strongly correlated with the ADCC activity specific for gp120 ($r = 0.511$, p-value < 0.0001) (Supplemental Figure 1B). However, there was no statistically significant association observed between gp41-specific ADCC activity and total proviral levels ($r = 0.110$, p-value $= 0.356$) (Figure 2.3A). Examining each individual proviral category, we again observed no significant association between gp41-specific ADCC activity and neither the size of the intact reservoir ($r = -0.0512$, p-value $= 0.686$) (Figure 2.3B) nor the number of defective proviral copies ($r = -0.109$, p-value $= 0.389$) (Figure 2.3C). Multivariate linear regression analyses controlling for potential confounding effects, as described above, for gp41-specific ADCC activity reported similar results (Supplementary Table 1). When the analyses were run with the inclusion criteria requiring viral suppression at the time of HIV DNA quantification as well as for six months prior, again we did not observe a significant change in the reported results (Pearson correlation: total: $r = 0.150$, p-value $= 0.223$; intact: $r = -0.0185$, p-value $= 0.887$; defective: $r = -0.0720$, p-value $= 0.578$).

Employing the previously described binning approach in relation to the cohort median gp41-specific ADCC activity, no significant difference was observed between the two groups for mean total (p-value $= 0.538$), intact (p-value $= 0.508$), or defective (p-value $= 0.531$) copies of HIV provirus (Figure 2.3D-F). When binning was performed based on a participant's relation to the cohort 75th and 25th percentile, there was no change in the observed statistical significance

for any of the proviral categories (total: p-value = 0.242; intact: p-value = 0.742; defective: p-value = 0.770) (Supplementary Figure 2.2D-F). Therefore, in contrast to what was observed with gp120-specific ADCC activity, ADCC activity against HIV Env gp41 at the time of ART initiation is not associated with levels of any proviral category. These contrasting findings suggest a unique role for ADCC-mediating antibodies specific for gp120 to impact the persistent proviral landscape during ART.

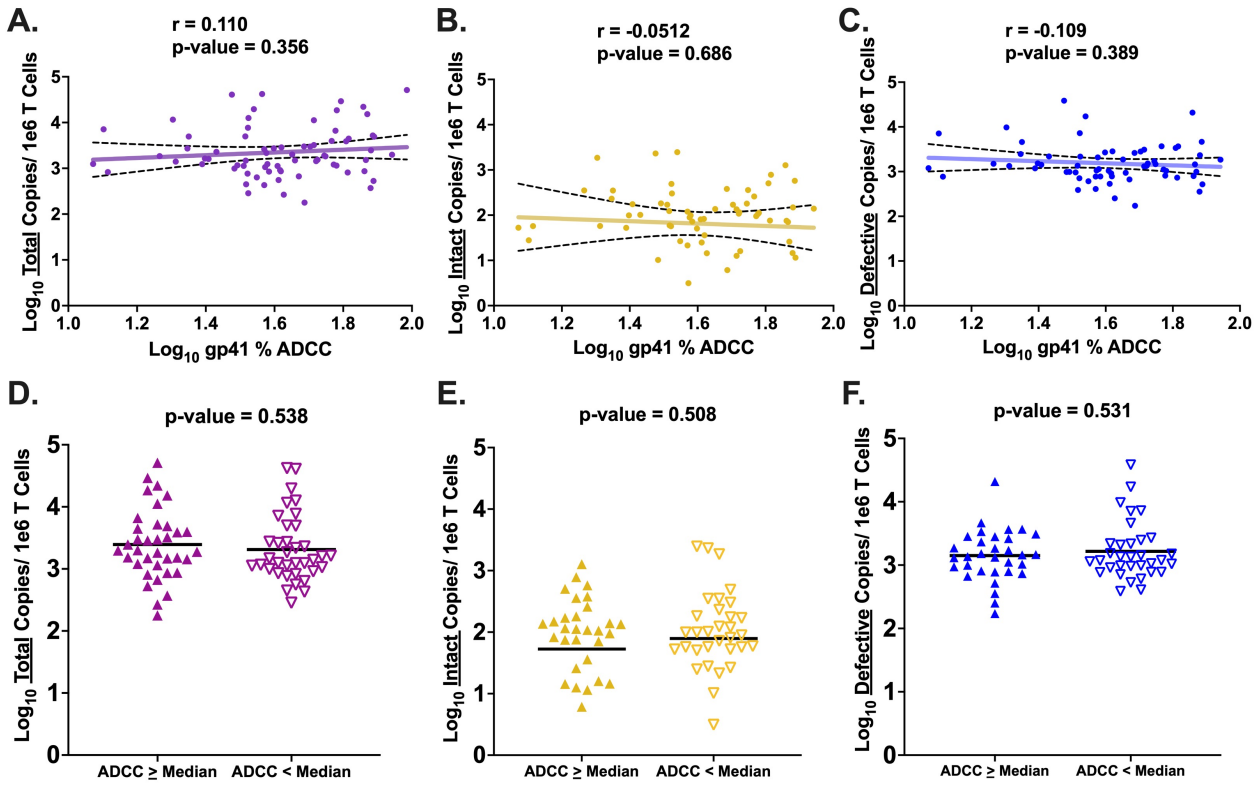


Figure 2.3 Association Between gp41-Specific ADCC Activity and Levels of HIV Provirus.

(A-C) Pearson correlation of $\log_{10}$ gp41-specific ADCC activity and copies of HIV provirus measured for each proviral category. The best fit line and its 95% confidence interval is imposed onto each graph. **(A)** Total HIV provirus (n = 72); **(B)** Intact HIV provirus (n = 68); **(C)** Defective HIV provirus (n = 68); **(D-F)** Unpaired t test comparing mean $\log_{10}$ HIV proviral copies between "ADCC > Median" and "ADCC < Median" groups. Participants were stratified into either group based on a participant's ADCC activity in relation to the cohort median; Participants at, or above, cohort median were labeled "ADCC > Median" with those below the cohort median labeled as "ADCC < Median." **(D)** Total HIV provirus (n = 72); **(E)** Intact HIV provirus (n = 68); **(F)** Defective HIV provirus (n = 68).

2.4 Discussion

The cytolytic nature of host immune-mediated effector functions such as ADCC supports the potential for antibody responses present prior to ART to impact the establishment of the HIV reservoir, yet our understanding of this remains limited. To address this, we assessed the association between pre-ART HIV Env-specific ADCC activity and levels of persistent HIV provirus. The results suggest a moderate inverse correlation between gp120-specific, but not gp41-specific, ADCC activity and the level of defective persistent provirus. The moderate nature of this observed association may reflect the fact that several factors presumably impact establishment of persistent HIV provirus, with our results implicating gp120-specific ADCC as one of these factors. Interestingly, we did not observe this same association with the size of the intact reservoir, which adds to the mounting evidence of potential differences in kinetics between these two proviral populations [39, 40, 390, 699].

HIV Env is presented on the surface of reactivated latent cells largely as gp120 or gp41 monomers [545] and is the main HIV protein targeted by ADCC-mediating antibodies [3, 340], with ADCC epitopes predominately exposed when Env binds CD4 within the same infected cell [3, 30, 340, 700]. Importantly, HIV uses its Nef and Vpu proteins to downmodulate cell surface levels of CD4, decreasing Env-CD4 interactions and limiting exposure of ADCC epitopes [140, 340, 701]. Thus, one hypothesis to explain the differences we observed in associations between gp120-specific ADCC and intact, versus defective, HIV provirus is that a defective provirus with nonfunctional Nef and/or Vpu could result in suboptimal CD4 downregulation, promoting exposure of Env-CD4-induced epitopes on a cell's surface and increasing susceptibility to ADCC-mediated clearance [702-704]. Studies probing the detailed structure of defective

proviruses and the corresponding functionality of their Nef and Vpu genes would be needed to test this hypothesis.

While epitope targets capable of mediating ADCC are found in both gp120 and gp41 subunits [705, 706], gp41-specific ADCC activity did not significantly correlate with levels of persistent provirus in our study. The Env trimer regularly sheds its gp120 subunit, leaving a gp41 stump displaying an immunodominant epitope on its ectodomain [707, 708] which is the primary antibody target during acute HIV infection [517, 708]. However, due to its highly variable nature, gp120 continually escapes antibody responses during chronic HIV infection promoting a broad polyclonal antibody response resulting in gp120 becoming the dominant antibody target [708-711]. This could similarly lead to increased ADCC activity targeting gp120 versus gp41. Thus, one hypothesis to explain the differences we observed in associations with HIV provirus levels and gp120-specific ADCC compared to gp41-specific ADCC activity is that there is a greater quantity, and quality, of antibodies targeting gp120 compared to gp41.

Our results demonstrating an inverse association between gp120-specific ADCC activity and levels of defective HIV provirus are particularly interesting given that ADCC-mediating antibodies specific for the V2 region in gp120 were identified as correlates of protection in the moderately successful RV144 vaccine trial [620]. This finding is notable as therapeutic vaccines intended to mediate clearance of cells harboring HIV provirus are currently beginning to enter clinical trials [712, 713]. The results of this study provide evidence to support consideration of ADCC activity as an immune outcome measure in these trials. While our observation that gp120-specific ADCC activity inversely correlates only with levels of defective provirus, an intervention capable of targeting cells with defective provirus could still prove useful in

decreasing the overall number of cells capable of contributing to chronic immune activation during long-term ART [20, 389, 391, 453, 661, 662].

There are several limitations to our study, the most relevant being that the investigated cohort included a wide age range at time of ART initiation. This is pertinent because for children living with untreated HIV infection, by the age of two there is approximately a 50% mortality rate [78, 676, 677], reaching 80% by the age of five [73]. Thus, the children in this study include the roughly 20% of children who lived past age five despite untreated HIV infection. Therefore, the cohort studied here may represent a unique population with less applicability to the broader population. This study also had several unique strengths, including access to pediatric samples beginning at the point of ART initiation. By leveraging the CS-IPDA, this study investigated intact and defective proviruses separately which is important as recent studies have demonstrated differences in population dynamics between these two proviral populations such as a significantly higher decay rate of intact proviruses compared to defective proviruses during long-term ART [39, 40, 699, 714]. Additionally, the CS-IPDA allowed for the study of a cohort from Kenya where HIV subtypes A and D are most prevalent, whereas subtype B has been the primary focus of most reservoir studies up to this point despite representing a relatively small fraction of the global HIV burden [675].

To date, the most relevant factor associated with the size of the established reservoir is time between primary HIV infection and ART initiation. Here, we observe gp120-specific ADCC activity inversely associates with the levels of defective HIV provirus during ART with a similar magnitude of association to that of time to ART initiation [450]. These findings suggest that host immune effector functions may limit the number of cells harboring defective HIV provirus during ART. Additionally, these data support the idea that the dynamics of cells harboring intact

HIV provirus differ from those harboring defective HIV provirus and thus should be studied, and treated, individually.

In summary, our data suggests that HIV gp120-specific antibodies capable of mediating ADCC may reduce the established levels of defective, but not intact, persistent HIV provirus. Importantly, this represents a factor that can be manipulated via biomedical interventions and thus could be of interest as a possible strategy to augment ART. Therefore, further studies on the impact of ADCC-mediating antibodies on levels of HIV provirus that persist during ART in larger, diverse cohorts are warranted.

2.5 Methods

2.5.1 Cohort

Between 2004 and 2005, the Pediatric Adherence Diary study enrolled ART-naïve children living with HIV in Nairobi, Kenya aged 18 months to 12 years in a longitudinal clinical trial to evaluate adherence diaries during ART [693]. The study provided ART at the enrollment visit to 103 children, and blood samples were collected at enrollment and again every three to six months during the first two years after ART initiation for those who remained in follow-up. The study was approved by the University of Washington and Fred Hutchinson Cancer Center Institutional Review Boards and Kenyatta National Hospital Ethics and Research Committee. Caregivers provided written informed consent for their children's participation and for the use of banked samples in future studies.

2.5.2 HIV RNA/DNA Measurement

HIV RNA was previously measured in longitudinal plasma samples using Gen-Probe HIV RNA assay with a lower-limit of detection of 150 copies/ml [715]. PBMC samples were selected for HIV DNA quantification from timepoints between 12 and 24 months post-ART initiation that had viral suppression (HIV RNA levels < 1000 copies/mL). From the original cohort (n = 103), samples that met this inclusion criteria were available for 72 participants, with up to two PBMC samples available per child. If two PBMC samples from the same child met the inclusion criteria, we quantified HIV provirus in both samples and averaged the two measures. To account for the diverse subtypes of HIV circulating in Kenya [675, 716], CS-IPDA [37] was used to measure the number of total and intact proviral copies isolated from cryopreserved PBMCs. Low-shearing genomic DNA extraction and CS-IPDA were performed as previously described [717]. CS-IPDA reactions were completed in triplicate with additional replicates performed on samples with no detectable intact provirus until either intact provirus was detected or a minimum of 105 cells were interrogated. The CS-IPDA can detect a single copy of intact provirus; samples with undetectable intact provirus were set to 0.5 copies over the number of cells interrogated normalized to 10 x 10^6 cells. In a prior analysis, less than 1% of intact sequences were incorrectly classified as defective, which suggests that underestimating intact provirus because of sequence diversity is rare [37]. The number of defective proviral copies was determined by subtracting the number of intact proviral copies from the total number of proviral copies. Intact provirus levels were only measured if samples had <40% DNA shearing as measured by the RPP30 reference assay [500, 717]. Thus, total provirus data were included in our analysis for all 72 participants, while intact and defective HIV proviral copies were included for 65 participants that had DNA shearing rates <40%.

2.5.3 Rapid and Fluorometric ADCC Assay

The Rapid and Fluorometric ADCC (RFADCC) assay, which has been associated with clinical outcomes [72, 581, 582] and is correlated with results from different ADCC assays [72], was performed as previously described [36, 72] to measure HIV Env-specific ADCC activity at study enrollment (time of ART initiation) using plasma samples heat inactivated at 56°C for 1 hour. Briefly, the cytosols of CEM.NKR cells (NIH AIDS Reagent Program, Catalog #458) were stained with CFSE dye (Vybrant CFDA-SE. Cell Tracer Kit, Invitrogen) followed by cellular membrane staining with either CellVue Claret Far Red cell Linker dye (Sigma Aldrich) or PKH26 cell linker dye (Sigma Aldrich). These double stained cells were then coated with either Clade A BG505.W6M.ENV.B1 gp120 (Cam-bridge Biologics; GenBank: ABA61515), Clade A/D BL035.W6M.ENV.C1 gp120 (Immune Tech; GenBank: DQ208480), or Clade C ZA.1197MB gp41 (Immune Tech; GenBank: AY463234) antigen at a ratio of 1.5ug of antigen per 100,000 cells for one hour at room temperature. The clade A BG505 gp120 antigen, which was derived from a Kenyan infant living with untreated HIV infection, is the dominant HIV clade circulating in Kenya [716] and the clade A/D recombinant BL035 gp120 antigen has previously been shown to be representative of gp120 from diverse clades when used in the RFADCC assay [582]. The ZA.1197MB gp41 antigen represents one of the few gp41 antigens derived from a primary isolate. During the one-hour coating step, plasma samples were diluted to either 1:100,000 if BG505 gp120 was the coating antigen, or 1:32,000 if BL035 gp120 or ZA.1197 gp41 was the coating antigen. These dilutions were experimentally determined to provide the best separation of measurements across samples from this specific cohort for each individual antigen while also avoiding a prozone effect. Additionally, monoclonal antibodies serving as positive controls were diluted to 100-500ng/mL and an Anti-HIV Immune Globulin

(HIVIG, NIH ARP, Catalog # 3957) positive control and a Human Negative Control Serum (NIH ARP, Catalog # 2411) were both diluted to a 1:5000 dilution. All samples were diluted in RPMI containing penicillin (100U/mL), streptomycin (100ug/mL), amphotericin B (250ng/mL), L-glutamine (2mM), and fetal bovine serum (10%) (RPMI complete). Following the one-hour antigen coating step, the double stained cells were washed and a total of 5,000 double-stained, coated target cells were added to 100ul of each plasma or control dilution in duplicate in a 96 well U bottom TC-treated plate (Corning). The target cells and plasma dilutions were mixed and then incubated for 15 minutes at room temperature, followed by the addition of 250,000 PBMCs from a seronegative donor for an effector to target cell ratio of 50:1. These cells were then left at 37^{o}C for four hours to allow for RFADCC activity to occur and then washed and fixed in 2% paraformaldehyde (Santa Cruz Biotechnology). The next day, RFADCC activity was measured via flow cytometry (BD Symphony). The CFSE, CellVue, and PKH26 dyes were detected in the FITC, APC, and PE channels, respectively. The collected data were then analyzed using FlowJo (v.9.9, Treestar). ADCC was determined as the percentage of either PKH or CellVue-positive, CFSE-negative cells out of the total PKH or CellVue-positive cells after subtracting for background activity. Background ADCC was determined as the ADCC activity of media against uncoated target cells which was set to 3-5%. All data was then normalized to the average ADCC activity measured in the HIVIG positive control wells. Three biological replicates, each consisting of two technical replicates, were performed for each antigen. For BG505 gp120 and ZA.1197 gp41 antigens, all three biological replicates were performed using seronegative PBMC donor cells from a different donor to assure that the results were not specific to a particular PBMC donor. The three biological replicates carried out for the BL035 gp120 antigen were performed using PBMCs from two different seronegative donors. The results for both BG505

and BL035 gp120 antigens across all replicates were averaged for each child to report one gp120-specific percent ADCC.

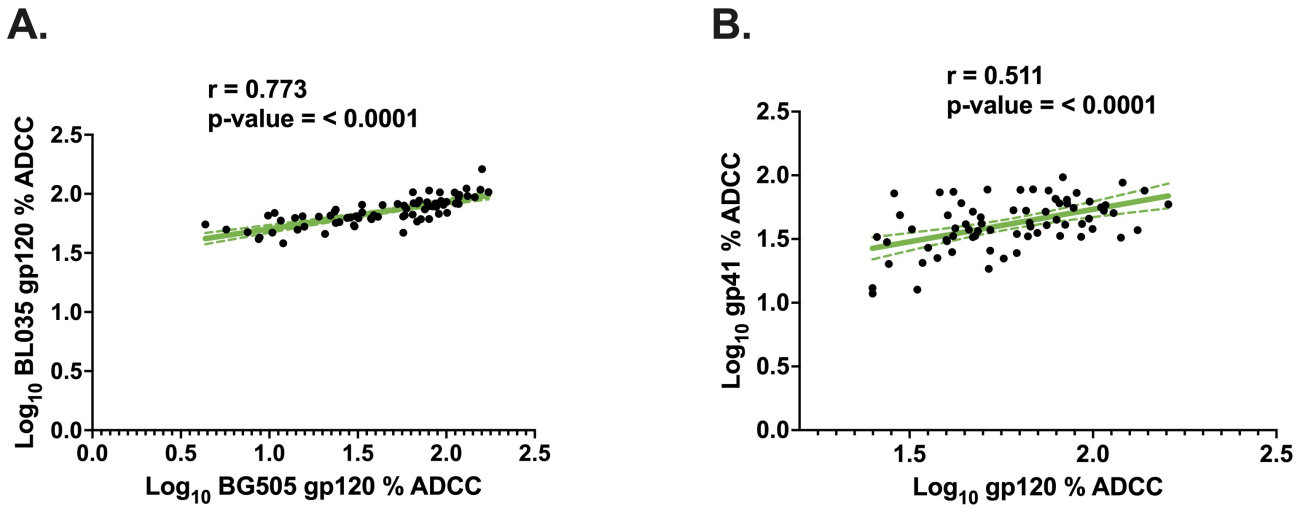
2.5.4 Statistical Methods

To reduce skewness, all HIV provirus and ADCC data were log transformed for analyses. Pearson correlation coefficients were generated to test for associations between each proviral category and both gp120 and gp41-specific ADCC activity. ADCC data was also stratified into two groups labeled either "ADCC > Median" or "ADCC < Median" based on a participant's plasma ADCC activity in relation to the cohort median. The ADCC data were additionally stratified into two groups labeled "ADCC High" for those in the highest quartile of ADCC activity or "ADCC Low" for those in the lowest quartile. Student's T-tests were performed to test for differences in the mean copy number of HIV provirus between ADCC groups. Multivariable linear regression models with backward stepwise selection were used to assess potential predictors of each category of HIV provirus and address confounding effects. Age at ART start, a proxy for time to ART, was included in the model given its established clinical significance. Other potential predictors included pre-ART CD4 percent, pre-ART viral load (log_{10} copies/mL), gp120 ADCC levels, and gp41 ADCC levels. A pre-determined cutoff for statistical significance was set at a p-value of < 0.05, with $0.05 <$ p-value < 0.01 deemed a trend. All analyses were performed using GraphPad Prism Version 9.5.0 or R version 4.0.4 (R Core Team 2021).

2.5.5 Cell Lines

In the RFADCC assay, CEM.NKR cells (RRID: CVCL_X622; originally derived from female human T-lymphoblastoid cells) were used as target cells. These cells were obtained from the NIH AIDS Reagent Program (cat #: 458) and maintained at 37ºC in RPMI 1640 media with penicillin (100U/mL), streptomycin (100ug/mL), amphotericin B (250ng/mL), L-glutamine (2mM), and fetal bovine serum (10%) added. We did not further authenticate the cells.

2.6 Supplementary Figures



Supplementary Figure 2.6.1. ADCC Measures Using Different Env Antigens are Highly Correlated.

(A) Pearson correlation of $\log_{10}$ ADCC activity measured against the Clade A BG505 gp120 antigen (x-axis) and the Clade A/D BL035 gp120 antigen (y-axis); **(B)** Pearson correlation of $\log_{10}$ gp120-specific ADCC activity (x-axis) and $\log_{10}$ gp41-specific ADCC activity (y-axis)

Supplementary Table 2.6.1. Linear Regression Models

Intact HIV Provirus	Mean (SD)	95% Confidence Interval	Univariate Coefficient (95% CI)	p-value	Full Model Multivariable Coefficient (95% CI)	p-value	Backward Selected Model Coefficient (95% CI)	p-value
Intact HIV Provirus (c/1e6 CD4 cells)	1.81 (1.03)	(1.55, 2.07)						
Age (Months) at Enrollment	64 (35)	(50.55, 77.45)	0.0040 (-0.0037, 0.0113)	0.3266				
HIV RNA (log_{10} c/mL)	5.84 (0.70)	(5.68, 6.00)	-0.0680 (-0.4555, 0.3196)	0.7323				
% CD4	10 (11)	(6.47, 13.54)	-0.0019 (-0.0259, 0.0221)	0.8761				
CD4 Count (cells/mm^3)	437 (459)	(308.81, 565.19)	-0.0004 (-0.0009, 0.0002)	0.2225				
Average % ADCC (gp120)	1.79 (0.19)	(1.77, 1.81)	-0.2012 (-1.5118, 1.1094)	0.7645	0.2354 (-1.3407, 1.8114)	0.7660	-0.0471 (-1.5270, 1.4328)*	0.9490
Average % ADCC (gp41)	1.62 (0.20)	(1.60, 1.64)	-0.2646 (-1.5404, 1.0112)	0.6857	-0.1641 (-1.6060, 1.2779)	0.8200	-0.1873 (-1.5810, 1.2064)*	0.7890

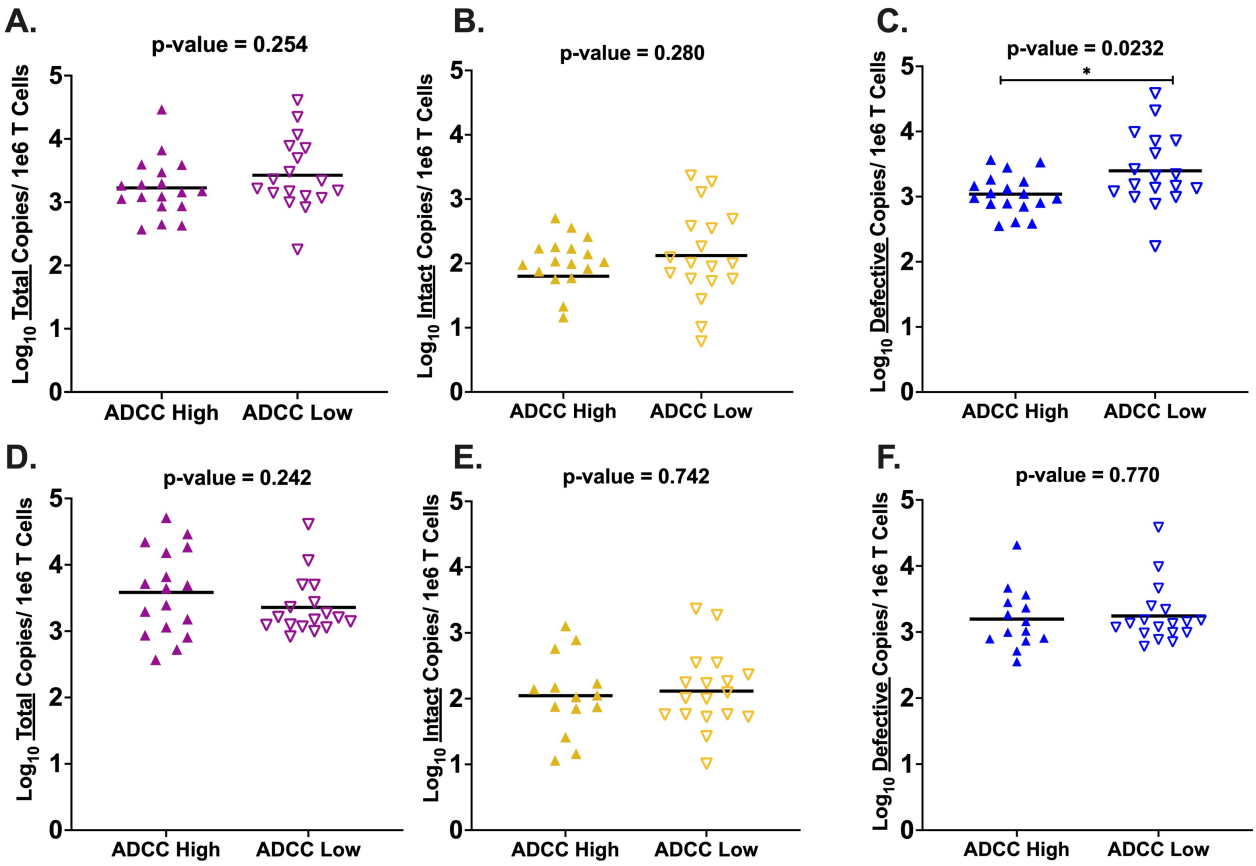
*Model includes only exposure of interest and age at enrollment (months).
**p-value <0.05

Total HIV Provirus	Mean (SD)	95% Confidence Interval	Univariate Coefficient (95% CI)	p-value	Full Model Multivariable Coefficient (95% CI)	p-value	Backward Selected Model Coefficient (95% CI)	p-value
Total HIV Provirus (c/1e6 CD4 cells)	3.35 (0.54)	(3.22, 3.48)						
Age (Months) at Enrollment	64 (35)	(50.55, 77.45)	0.0021 (-0.0016, 0.0058)	0.2677				
HIV RNA (log_{10} c/mL)	5.84 (0.70)	(5.68, 6.00)	-0.1221 (-0.3087, 0.0645)	0.2050				
% CD4	10 (11)	(6.47, 13.54)	0.0021 (-0.0100, 0.0142)	0.7391				
CD4 Count (cells/mm^3)	437 (459)	(308.81, 565.19)	-3.3e-05 (-0.0003, 0.0003)	0.8190				
Average % ADCC (gp120)	1.79 (0.19)	(1.77, 1.81)	-0.6485 (-1.3103, -0.0134)	0.0589	-0.6560 (-1.4260, 0.1139)	0.0935	-0.5137 (-1.2452, 0.2177)*	0.1650
Average % ADCC (gp41)	1.62 (0.20)	(1.60, 1.64)	0.2995 (-0.3355, 0.9345)	0.3585	0.2278 (-0.4790, 0.9354)	0.5221	0.3250 (-0.3474, 0.9975)*	0.3380

*Model includes only exposure of interest and age at enrollment (months).
**p-value <0.05

Defective HIV Provirus	Mean (SD)	95% Confidence Interval	Univariate Coefficient (95% CI)	p-value	Full Model Multivariable Coefficient (95% CI)	p-value	Backward Selected Model Coefficient (95% CI)	p-value
Defective HIV Provirus (c/1e6 CD4 cells)	3.18 (0.43)	(3.08, 3.28)						
Age (Months) at Enrollment	64 (35)	(50.55, 77.45)	0.0018 (-0.0013, 0.0049)	0.2660				
HIV RNA (log_{10} c/mL)	5.84 (0.70)	(5.68, 6.00)	-0.0340 (-0.1922, 0.1243)	0.6755				
% CD4	10 (11)	(6.47, 13.54)	0.0004 (-0.0094, 0.0103)	0.9288				
CD4 Count (cells/mm^3)	437 (459)	(308.81, 565.19)	-9.1e-05 (-0.0003, 0.0001)	0.4501				
Average % ADCC (gp120)	1.79 (0.19)	(1.77, 1.81)	-0.6349 (-1.1570, -0.1129)	0.0202**	-0.0515 (-1.1430, 0.1129)	0.106	-0.5882 (-1.1526, -0.0239)*	0.0414**
Average % ADCC (gp41)	1.62 (0.20)	(1.60, 1.64)	-0.2336 (-0.7614, 0.2942)	0.3890	-0.2245 (-0.8100, 0.3611)	0.4450	-0.2146 (-0.7784, 0.3493)*	0.4490

*Model includes only exposure of interest and age at enrollment (months).
**p-value <0.05



Supplementary Figure 2.6.2. Comparing HIV Provirus Levels in ADCC High Versus Low Groups.

Unpaired t test of $\log_{10}$ mean HIV proviral copies between "ADCC Strong" and "ADCC Weak" groups. Participants with ADCC activity at, or above, the cohort 75th percentile were labeled "ADCC Strong" (n = 18) with those at, or below, the cohort 25th percentile labeled as "ADCC Weak" (n = 18). **(A-C)** Stratification based on gp120-specific ADCC activity. **(A)** Total HIV provirus; **(B)** Intact HIV provirus; **(C)** Defective HIV provirus; **(D-F)** Stratification based on gp41-specific ADCC activity. **(D)** Total HIV provirus; **(E)** Intact HIV provirus; **(F)** Defective HIV provirus. *denotes a p-value ≤ 0.05

Chapter 3. Conclusions and Future Directions

The reservoir of cells that harbor transcriptionally silent HIV provirus capable of stochastic reactivation [13] that persists on antiretroviral therapy (ART) is the main barrier to achieving a cure for HIV [9, 10]. While ART can allow for a person living with HIV to live a near-normal lifespan [1], chronic HIV infection, even when virally suppressed, is associated with chronic immune activation [648, 650, 718] which correlates with an array of adverse health outcomes [212, 651-659]. The stochastic reactivation of latent HIV provirus likely contributes to this chronic immune activation, whether the provirus is intact or defective [20, 389, 391, 592, 650, 661, 663, 718]. As such, several clinical benefits associated with smaller reservoir size have been reported, including decreased chronic immune activation and a prolonged time to viral rebound upon cessation of ART [130, 665]. Therefore, identifying factors associated with a reduced reservoir size could be important for informing future clinical interventions.

The reservoir invariably decays very slowly on ART, though the size of the reservoir can vary significantly from person to person [109, 501]. To date, very few determinants of reservoir size have been described, however. In chapter 2, HIV reservoir measurements among a cohort of children after one year of continuous ART was described using the cross-subtype intact proviral DNA assay. Importantly, this cohort was a Kenyan cohort representing non-subtype B HIV, which is important because studies of non-B subtypes have been underrepresented in reservoir studies to date in relation to its global disease burden.

Using plasma samples from the children in this cohort that were collected just prior to ART initiation, pre-ART ADCC activity was also measured, allowing for the investigation of the relationship between ADCC and the size of the persistent reservoir established soon after ART initiation. The study demonstrated a moderate, inverse association between pre-ART HIV Env

gp120-specific ADCC activity and the levels of defective provirus that persist on ART in the children in the cohort. Not only did this finding identify a factor associated with reservoir size that is capable of being augmented [620] via biomedical interventions such as through vaccination [620] or passive transfer of bNAbs [640], but it also provides evidence to support the long-held assumption that the host immune response is capable of impacting cells of the latent reservoir. The fact that this association was specific for antibodies that target gp120, and only with levels of defective provirus but not the intact reservoir, opens the possibility for many follow up studies to explore this relationship further.

3.1 Accumulating Further Evidence of Association Between ADCC and HIV Reservoir Composition

A limitation of the study described in chapter 2 is the fact that the investigated cohort consisted of children with a median age of almost five years. This is pertinent because children living with untreated HIV face a mortality rate of 80% by this age [73], suggesting that the children in this study may represent a cohort with unique immune properties that may not be applicable to the wider population. With respect to ADCC, studies have demonstrated that higher ADCC activity in maternally transferred antibodies in infants [70, 72, 582] as well as *de novo* antibodies [613, 719] directly correlates with survival. This is also demonstrated by observations that long-term non-progressors tend to have higher ADCC activity than people who are typical progressors [720, 721]. Thus, this cohort may represent atypical levels of ADCC if they largely represent the 20% of children who survived to the age of five with untreated HIV infection. Additionally, children living with untreated HIV infection have repeatedly been shown to develop bNAbs faster and more frequently than adults [93, 95, 96, 98], therefore the cohort studied

here may also consist of many children with a bNAb response which could potentially influence the ability of their circulating plasma antibodies to mediate ADCC [722, 723].

To test that the findings of this study are applicable to adults, this study should be repeated in a cohort of adults who initiated ART during chronic infection. An advantage to an adult study is that a greater volume of blood can be obtained from adults than from children, permitting more analytical testing to be performed. Whereas with children, there is a limited quantity of available PBMCs per timepoint because less blood can be drawn at any one given time. With more PBMCs available from an adult cohort, the investigation of the relationship between Env-specific ADCC and the composition of the HIV reservoir can be performed in more detail by allowing for further tests such as FLIPS to be performed [394]. This would allow for sequence analysis of reservoir cells to be performed which could lead to studies that aimed at understanding if certain viral genetic factors such as defects in *nef* or *vpu* impact the observed association between ADCC activity and the defective reservoir.

Due to limited sample volumes derived from children, the number of possible CS-IPDA repeats that can be performed for an individual from a certain timepoint is smaller. This matters in the case of a failed CS-IPDA run or if there is too great of genomic DNA shearing in a sample to be able to estimate intact provirus, as the number of possible repeats to overcome these failures is limited with limited sample availability. Thus, with an adult cohort, the sample size of CS-IPDA data for an individual may be able to be increased by allowing for more opportunities for assay repeat runs in case of failed CS-IPDA runs or if a sample is unable to be used to estimate intact reservoir size due to too high of genomic shearing in sample processing. This would allow for an increase in statistical power to identify significant correlations between ADCC and reservoir size.

Additionally, reservoir measurements obtained via CS-IPDA requires viral suppression at the time from which the sample was collected and, ideally, for six months prior to that timepoint, as well. In a pediatric cohort this requirement is more challenging to meet because children and adolescents tend to have a more difficult time consistently adhering to their ART regimen compared to adults [724]. Thus, in an adult cohort, more of the samples obtained may meet the requirements for a proper CS-IPDA measurement which could represent another way that sample size, and subsequently statistical power, may be increased if this study were to be repeated in adults.

A major limitation of the study performed in section 2 is that ADCC activity was measured against cells coated with heterologous, monomer, recombinant Env proteins which limits its physiological relevance [141, 340]. This method, therefore, is unable to measure the ADCC activity of antibodies specific for conformational epitopes. The use of heterologous recombinant Env, too, may lose detection of ADCC mediated by antibodies that may not recognize the amino acid sequences of the specific recombinant Env used in the assay.

Therefore, if this study were to be repeated in an adult cohort, testing for the ability of an individual's plasma antibodies to facilitate ADCC-mediated killing of autologous CD4+ T cells [725] may provide a more physiologically relevant ADCC measure. With greater sample availability from an adult cohort, there would likely be sufficient PBMCs to perform both the CS-IPDA and an autologous ADCC killing assay. By demonstrating the ability of an individual's antibodies to mediate ADCC against autologous HIV Env, the conclusion that ADCC can impact the level of cells harboring HIV provirus via clearance would be greatly bolstered.

To add further evidence to the claims that ADCC can alter the size and composition of the HIV reservoir, pre-ART ADCC activity should also be examined for association with the rate of

longitudinal reservoir decay. Although reservoir decay is generally quite slow over time consistently across individuals [121, 442, 501], it has been reported that certain factors can impact the speed at which it occurs. Documented instances of viral blips while on ART have been reported to be associated with a longer reservoir half-life compared to those who have not experienced a viral blip [109, 501, 726]. This indicates that while there is generally little variation in reservoir decay within an overall population, there are certain factors that can still impact it, meaning there may also possibly be more yet to be identified.

If increased ADCC activity specific for gp120 is influencing a reduction in defective provirus levels at reservoir establishment via ADCC-mediated clearance, then one might hypothesize this would continue during long-term ART. Therefore, by measuring the reservoir by CS-IPDA at several longitudinal timepoints past one-to-two years of long-term ART, the reservoir half-life and rate of decay can be estimated for an individual. The relationship between this measure and HIV Env-specific ADCC activity could then be evaluated to further contribute to our understanding of the impact ADCC can have on the persistent HIV reservoir. ADCC measures in this case should be performed at a pre-ART timepoint in addition to several timepoints during continuous ART to evaluate if, or how much, ADCC activity remains in the absence of viremia. This could be evaluated for both the pediatric cohort described in chapter 2, as well as the theoretical adult cohort proposed in this chapter.

3.2 Evaluating the Role of *nef* and *vpu* in the Differences Observed Between Intact Versus Defective Provirus Levels

The results presented in chapter 2 reported that pre-ART gp120-specific ADCC activity inversely correlated with the levels of defective, but not intact, provirus that persists on ART. It was speculated that this may be due to defects in *nef* or *vpu* in defective proviruses compared to intact proviruses which presumably contain functional versions of these genes. *Nef* and *vpu* promote downregulation of CD4 expression on the surface of infected host cells, and several studies have reported that this decrease in CD4 on the cell surface limits interactions between CD4 and Env on an infected cell, keeping the Env trimer in a closed state compared to an open conformation when Env is bound to CD4 [3, 140, 141, 340]. By keeping the Env trimer closed, this occludes antibody binding to epitopes known to promote ADCC, many of which are induced by CD4 binding [140, 701].

Therefore, if a cell harboring a defective provirus has a nonfunctional *nef* and/or *vpu* gene, then upon reactivation and Env production, the Env presented on the cell surface would be more likely to bind to cell surface CD4, opening up its trimer and exposing ADCC-mediating epitopes [3, 140, 141, 340]. This could then result in an increased likelihood of ADCC-mediated clearance and possibly explain why there is a relationship observed between gp120-specific ADCC and defective, but not intact, provirus levels. In future directions of the study described in chapter 2, this theory should be evaluated.

To evaluate this hypothesis *in vitro*, multiple stable cell lines that contain an integrated HIV provirus similar to existing J-lat cells, which are a T lymphocyte cell line with a stably integrated HIV provirus [483, 727], could be generated. More specifically, the generated cell lines should include one with a full-length, wildtype provirus, one with a full-length provirus with a

nef deletion (ΔNef), one with a full-length provirus with a *vpu* deletion (ΔVpu), and one with a full-length provirus with both a *nef* and *vpu* deletion (ΔVpu/ΔNef). Many existing J-lat cell line latency models have a *nef* gene replaced with a reporter gene such as GFP [483], so for the purposes of this study it would be best to generate a new version of the cell line model to more specifically test the impacts of *nef* and *vpu* deletions on a reactivated cell's ADCC susceptibility.

Reactivation of the generated cell lines in parallel using a latency reversal agent could then be used to induce cell surface Env expression [728]. After viral transcription was induced in each respective cell line, plasma samples from a cohort with previously characterized ADCC activity such as the one described in section 2 could then be added to the reactivated cells. Following incubation with the plasma samples, PBMCs from a seronegative donor could then be added to allow for ADCC to be carried out. To quantify ADCC-mediated killing, the cells could be stained with a viability dye before ADCC was induced, therefore percent killing could be measured by quantifying the percentage of dead cells within each condition via flow cytometry compared to a negative control.

A significantly increased percentage of cell death observed in the ΔNef, ΔVpu, and ΔVpu/ΔNef conditions would be expected compared to the wildtype control. Based on studies demonstrating that *vpu* cannot compensate for a loss of *nef* in promoting ADCC evasion [7], however, we may expect to see a greater increase in ADCC-mediated killing in the ΔNef and ΔVpu/ΔNef conditions compared to the ΔVpu condition, alone. We also would expect that the ADCC activity exhibited by each individual plasma sample would correlate well with its ADCC activity measured in whatever assay was used to previously characterize the plasma's ADCC activity.

The use of an *in vivo* model could also be used to test this hypothesis. Because the effects of HIV *nef* and *vpu* have been shown to be species-specific [729, 730], the use of NHPs would not be ideal for investigating the effects of these proteins on ADCC susceptibility. Therefore, a humanized mouse model would be best-suited for this investigation. Using PBMCs from a seronegative donor, susceptible target cells could be injected into mice with functionally active NK cells [142] to produce a mouse model of HIV infection. A primary HIV strain would be used to establish infection and untreated infection would be allowed to progress for three months to allow for reservoir establishment to occur [731].

The mice would then be broken up into four groups with one group started on ART alone, one group started on ART and co-administered plasma derived from people with known high gp120-specifc ADCC activity, another group started on ART and co-administered plasma derived from people with known low gp120-specifc ADCC activity, and finally another group started on ART and co-administered HIV seronegative plasma. All mice would continuously have their viral loads monitored, with the goal of inducing an undetectable viral load. For mice receiving human plasma, repeated doses of plasma would be administered once a week for the remainder of the study.

After three months of an undetectable viral load, the mice would then be bled to harvest all obtainable PBMCs for reservoir analysis. The intact and defective provirus levels could then be evaluated for each mouse in each group using IPDA. We would expect to see in the group only treated with ART and the group receiving ART and HIV seronegative plasma to have the highest average levels of defective provirus, followed by the group started on ART with low ADCC-mediating plasma co-administration, with the group receiving ART and high ADCC-mediating plasma to have the lowest levels of defective provirus.

The results observed in the study described in chapter 2 of this thesis added to the growing literature describing differences in reservoir dynamics between cells harboring intact versus defective provirus [39, 40, 714]. However, the mechanism behind the findings or their applicability beyond pediatric HIV reservoir is yet to be understood. Therefore, by repeating the study in a cohort of adults, the applicability of the findings outside of pediatric HIV could be discerned. This could additionally elucidate mechanistic understandings by allowing for further testing to be done thanks to the increased volume of blood collected from adults compared to children. With more PBMCs to work with, the assessment of provirus sequence analyses like those gleaned from the FLIPS assay could be performed, allowing to test hypotheses regarding *nef* and *vpu* function and how they relate to antibody-mediated reservoir clearance *in vivo*.

The mechanism could also be further understood using *in vitro* and *in vivo* models where certain factors such as particular viral gene influences can be better manipulated and controlled. If the results of these proposed experiments supported the hypothesis that gp120-specific ADCC-mediating antibodies are capable of mediating clearance of reactivated reservoir cells that express Env in an "open" state, this could have a potential influence on future cure strategies. In alignment with studies that have shown that CD4 mimetic compounds can sensitize reactivated reservoir cells to ADCC-mediated killing by inducing Env to take on an "open" trimer state [30, 142], the results of the experiments proposed in this section could suggest that adding CD4 mimetic compounds and passive transfer of gp120-specific bNAbs capable of mediating ADCC to standard ART may promote clearance of both the intact and defective reservoir by a person's immune system.

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