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Characterization of HIV-1 Superinfection in Cohabiting Heterosexual African Couples

By

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Graduate Division of Biological and Biomedical Science
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Characterization of HIV-1 Superinfection in Cohabiting Heterosexual African Couples

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M.S., Purdue University, 2008
B.S., Indiana University, 2007

Advisor: Eric Hunter, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Science
Immunology and Molecular Pathogenesis
2013

Abstract

Characterization of HIV-1 Superinfection in Cohabiting Heterosexual African Couples
By Debby Basu

Human immunodeficiency virus type 1 (HIV-1), responsible for the AIDS pandemic, accounts for a profound global health burden, the heaviest of which is on Sub-Saharan Africa, in which most infections are genotypically identified as Subtype C. Despite major successes in public health interventions and anti-retroviral treatment, HIV-1 continues to accrue new infections worldwide, imploring an effective and cross-protective HIV-1 vaccine. To determine potential immunologic correlates of a protective HIV-1 vaccine, we investigated situations of natural re-infection, or superinfection, by HIV-1. Identifying factors affecting susceptibility to superinfection may elucidate protective factors that will be necessary to elicit in an HIV-1 vaccine.

Here we identified three cases of intrasubtype C superinfection in a cohort of twenty-two epidemiologically unlinked Zambian couples. We found that, despite longitudinal viral sequencing and phylogenetic analysis, all three cases of superinfection occurred from non-spousal partners within the first year of infection. This suggested that both sexual risk behavior and early immune responses might play a potential role in superinfection. Full-length envelope (*env*) sequencing data also surprisingly suggested very limited diversification of the *env* gene prior to superinfection, again supporting the hypothesis of limited neutralizing antibody (Nab) pressure on the virus to adapt, and a potential defect in the humoral response prior to superinfection.

We compared neutralizing and binding antibody responses within the first year of infection in the three superinfected individuals against 10 matched non-superinfected controls. Here we show that the superinfected group exhibited statistically significantly lower levels of autologous neutralizing antibodies to their founder/early viruses prior to superinfection as compared to Nab responses from non-superinfected controls at similar time points. Binding IgG antibodies to Subtype C gp120 and V1V2 proteins also trended towards being lower prior to superinfection. Together, this suggested that risk of superinfection might be highest during early infection, but that strong IgG antibodies (both neutralizing and potentially non-neutralizing) could play a role in protection from superinfection during this period. These data suggest that primary infection may elicit some protection against intrasubtype superinfection and lend hope towards the feasibility of a regionally-based HIV-1 vaccine in areas where a single subtype predominates.

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Dedication

In memory of my grandfather, Nani Gopal Bakshi, who believed in me so much that he started calling me Dr. Debby Basu during my first year of graduate school.

Acknowledgements

I would like to thank:

- My parents, Minakshi and Dipan Basu, for their unlimited love and support
- My brother, Joey, for always believing in me and making me laugh
- Eric for being the most generous, inspiring, encouraging and coolest mentor
- Dave for reminding me daily what the big picture is
- My BFFs (SJ, VS, SW, CB, MD, JP) for always keeping me happy and grounded
- Jessica and Dan for being my "wolf pack" from our first day in the lab
- Cindy and Megan for being my antibody response coaches
- Colleen, Naina and Hope for being my superinfection support system
- The entire Hunter lab and personnel for making lab my home
- Effie, for every single appointment on Eric's calendar and all the secret chocolate
- Finky, Alfred and Shayla for reminding me regularly why we are doing this
- My entire thesis committee for their invaluable source of guidance
- Drs. William Kilembe, Etienne Karita, Jean Bizimana and their lab staffs at Projet San Francisco and the Zambia-Emory HIV Research Project for reminding me how much I really love what I do and inspiring me to want to do it better
- Dr. Susan Allen and the entire RZHRG staff for their tireless work toward establishing and maintaining the RZHRG cohorts
- All of the human volunteers for their truly invaluable contribution to this body of work and the progress made in this field

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Chapter 1: Introduction

Global Burden of HIV/AIDS

A two-page report from the CDC in 1981 described the first clinical cases of Acquired Immunodeficiency Syndrome (AIDS) symptoms in 5 men who have sex with men (MSM) in the United States [1]. Sadly, the number of individuals infected by the etiologic agent of AIDS, Human Immunodeficiency Virus type 1 (HIV-1), identified in 1983 [2, 3], has increased exponentially since this time and has resulted in a huge global burden of health. UNAIDS predicts that over 34 million individuals were living with HIV, and 1.7 million people died from AIDS-related causes in 2011 [4]. Therefore, the need for an effective prophylactic HIV-1 vaccine continues to be critically important to address this profound global heath crisis. The primary modes of HIV-1 transmission are through unprotected sexual contact, percutaneous exposure and perinatal transmission [5]. Target risk groups include MSM, heterosexual couples [6], injection drug users, commercial sex workers and children born from HIV+ mothers [4].

Despite a promising decline in the incidence of new infections in the last decade in Sub-Saharan Africa, this region still accounted for approximately 1.8 million new infections in adults and children as of 2012 and has suffered the heaviest health burden from HIV/AIDS, with approximately 70% of global HIV infections occurring in this region [4]. Tackling the AIDS pandemic has been complicated by a multitude of factors, including both biological ones, such as the virus's unprecedented sequence diversity, its capacity to deplete critical immune cells and its ability to escape immune pressure, and social ones, including socioeconomic status, stigma associated with the disease and access to antiretroviral treatment in vulnerable populations.

Origin and Classification of HIV-1

Human immunodeficiency virus (HIV), from the *Lentivirus* genus and *Retroviridae* family, is an approximately 9kb single-stranded, positive-sense enveloped RNA virus that encodes 9 genes (gag, pol, env, vpu, tat, rev, nef, vif, vpr). These genes are reverse transcribed by an error-prone, but multi-functional viral enzyme called reverse transcriptase (RT) that is present in the virion along with two copies of the viral genome (pseudodiploid). RT has four catalytic activities including being a DNA and RNA-dependent DNA polymerase, helicase and RNase H (endo and exonuclease functions), all of which are critical for reverse transcription of the viral RNA genome into a double-stranded proviral DNA that can be integrated, through the action of the virus's integrase enzyme, into the host chromosome for subsequent virus production.

Recombination between the co-packaged genomic templates has been shown to occur during reverse transcription [7, 8]. This can also occur between genomic RNA templates of different proviruses present in a multiply-infected cell [7], however the probability of forming the genomic RNA heterodimer required for recombination may be lower if sequences are too divergent [8]. The predominant "copy choice" model of recombination occurs during the conversion of positive sense genomic RNA template into the antisense template by RT, and occurs when the RT enzyme switches between RNA templates and copies parts of both templates as it moves to the 5' end of the positive sense template [8, 9]. Another model of recombination occurring through strand displacement during the subsequent step of reverse transcription called second strand (or + strand) synthesis can also occur; however, it is considered to contribute less to generation of recombinants [8]. The HIV viral genome is considered pseudodiploid because even though there may be two distinct genomes co-packaged into a single virion, only one intact copy (potentially a recombinant species) will be produced and integrated

as proviral DNA, and upon subsequent genomic RNA synthesis, two copies of this viral genome will be packaged into virions [9].

Gag, *pol* and *env* genes are responsible for major viral functions including *gag*'s critical role in defining viral structure and *po*l's enzymatic activity (including integrase, protease and reverse transcriptase). The 2.5-3kb *env* gene encodes a 160kDa polyprotein gp160, three of which assemble into a trimeric structure in the endoplasmic reticulum. Each gp160 is subsequently cleaved, in the Golgi complex, into two glycoproteins, gp120 and gp41. These non-covalently associated heterodimers thus form the trimeric spikes embedded into the viral envelope on the surface of virions that facilitate viral entry and fusion with target cells. The other six genes are considered accessory genes, but play important roles enhancing viral infectivity, evading host immune recognition, modulating viral genome transcription and replication, and facilitating virion budding and release at the plasma membrane.

HIV is subdivided into HIV-1 and HIV-2 variants, each of which was distinctly transmitted through cross-species transmission events from non-human primates infected with simian immunodeficiency viruses (SIV) [10-13]. HIV-1 has 4 distinct groups ("Main" or M, N, O, P) that have been categorized phylogenetically by viral sequencing. Similarly, each of these groups was transmitted to humans by an independent cross-species transmission event from non-human primates. HIV-1 Group M was transmitted by SIV_{cpz}-infected *Pan troglodytes troglodytes* chimpanzees in West Central Africa [10, 12, 14] likely during the early 1900s [15, 16], further diversified in this region [14, 16, 17] and is responsible for the AIDS pandemic which emerged in the early 1980s. HIV-2, which has been subcategorized phylogenetically into 8 groups A-H, and HIV-1 groups N-P have had a relatively limited global burden with a narrow geographic scope, mainly affecting individuals in West Africa (HIV-2 group A and B) [18, 19] and Cameroon (HIV-1 groups O and P)[20-24]. HIV-1 Group M is subcategorized into 9

phylogenetically distinct “pure” subtypes (A-D, F-H, J-K), which have variable phylogeographic distribution worldwide, although about half of all global HIV-1 infections are subtype C [25].

Viral Diversification

Due to the virus’s error-prone reverse transcriptase enzyme (contributing to a complex intrahost quasispecies) [26], rapid rate of viral replication [27, 28] and recombination [8, 9, 25, 29-31], sequence diversity of HIV-1 across global primary HIV-1 isolates is extraordinary, with intrasubtype and intersubtype sequence variation being around 8-17% and 17-35%, respectively [30, 32, 33]. Host factors such as the natural error rate of the host RNA polymerase enzyme and cytodine deamination in the negative strand of the viral genome caused by the host viral restriction factor Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like (APOBEC3) can also contribute towards accumulation of sequence diversity [34]. Through mathematical modeling of sequence evolution [35] as well as molecular characterization of single viral genomes through single genome amplification (SGA) and 454 deep sequencing [36, 37], it has been shown that even within a single host, viral sequence diversification occurs early post-infection in accord with establishment of viral set point [38]. This diversification reflects the temporal accumulation of synonymous and non-synonymous mutations, often associated with waves of escape from both neutralizing antibody (Nab) and CD8+ cytotoxic T lymphocyte (CTL) pressure [36-46].

Because retroviruses encapsidate two copies of genomic RNA, if a cell is infected with two genetically distinct variants, the virus can package an RNA representing each. Following subsequent infection, and during reverse transcription, therefore, a recombinant DNA provirus can be produced that represents a mixture of both parents. Recombination can potentially result in acquisition of immune escape or drug resistance

mutations conferred by a parental viral strain, as well as dramatic genetic diversity of the viral quasispecies in individuals infected with more than one HIV-1 variant by way of dual HIV-1 infection [47-52]. Depending on whether two parental HIV-1 viruses of the same or different subtypes recombine, the resulting virus can be an intrasubtype or intersubtype recombinant species, respectively. Furthermore, if an intersubtype recombinant virus is productively packaged into virions and transmitted to a new host, this could lead to the transmission of a circulating recombinant form (CRF).

Both CRFs, which represent intersubtype recombinants found in at least three epidemiologically unlinked individuals [53], and unique recombinant forms (URFs), representing recombinants with fewer identified isolates, are widely distributed globally [30, 31, 54]. CRFs have been recognized to account for approximately 10% of all HIV-1 infections worldwide [55]. Therefore recombination between two distinct HIV-1 variants in an infected individual can be attributed as one of the major factors contributing to overall global diversity of HIV-1 variants. Interestingly, Kinshasa in the Democratic Republic of Congo (DRC), which has been considered an epicenter of early HIV-1 diversification and spread, has intra-country HIV-1 sequence diversity comparable to that of the world [54], in which all pure subtypes, many CRFs, URFs and rare, unclassified variants are represented [14].

HIV-1 Pathogenesis and Immunopathology

Using the dynamic viral Env glycoprotein gp120, HIV-1 preferentially binds to and infects target cells expressing the CD4 receptor and CCR5 or CXCR4 co-receptor, which are primarily CD4+ T lymphocytes and macrophages [56-62]. The ability of the virus to integrate into the host cellular genome, via the integrase enzyme, is an important mechanism by which the virus is able to usurp cellular machinery for transcribing its genome and potentially remain undetected in a transcriptionally silent cellular reservoir

[63]. The virus establishes chronic disease in hosts through depletion of mucosal CD4+ cells [64-69], hyperimmune activation via microbial translocation [64, 65, 70-72] and multiple complex methods of escape from both cellular and humoral arms of the immune system [38]. Though the hallmark clinical endpoint of AIDS disease is CD4+ T cell loss, the B cell compartment (responsible for the establishment of antibody-mediated immunity) has also been shown to exhibit substantial abnormalities as an effect of chronic immune activation and persistent HIV-1 infection. Some of these effects include polyclonal activation and hypergammaglobulinemia [73], alterations and dysfunction in specific B cell compartments [74-76]. In addition, germinal centers, the sites in secondary lymphoid organs in which B cells mature and high affinity antibodies are generated and refined, are also severely depleted during early HIV-1 infection [77].

CD8+ cytotoxic T lymphocytes (CTL) have been associated with reduction in set point viral load in the newly-infected host and viral control in HIV-infected individuals [38, 78-80]. Therefore, viral escape mutations from CTL recognition commonly appear in the viral population after the initial immune response [38, 81]. Although cellular responses are critical for control of viremia, passive antibody studies in animal models, in addition to recent immunological correlates analyses from the RV144 vaccine efficacy trial, have suggested that the humoral (antibody) arm of the immune system could contribute towards protection from primary infection via an HIV-1 vaccine [82-96].

Realistically, it appears evident that a successfully protective and sustained HIV-vaccine mediated immune response must engage both strong protective antibody and T-cell mediated immune responses. However, correlates of immune protection based on evidence from human studies have historically been a challenge to identify. Evidence of HIV re-infection or superinfection is commonly seen, indicating that the primary immune response elicited in these cases were insufficient at protecting against secondary HIV-1 infection [55]. In addition, it must be recognized that under normal circumstances

and even with anti-retroviral treatment (ART), there is no record of an HIV+ individual successfully and completely clearing the virus. Finally, to date, only one human vaccine efficacy trial (RV144) has yielded any evidence of HIV-1 vaccine-mediated immune protection via antibody-based protection [87, 92, 93, 97]; however, this study importantly does give some proof-of-concept that a prophylactic HIV-1 vaccine may be possible.

Heterosexual Transmission of HIV-1

Despite the fact that transmission risk is lowest in heterosexual exposure to HIV-1 amongst all other major transmission routes (e.g. MSM, mother-to-child, percutaneous inoculation, etc.), heterosexual transmission of virus through the genital mucosal barrier still accounts for the majority of HIV-1 infections worldwide [5, 98]. Conservative estimates suggest that the probability of a transmission per exposure event at the female genital tract is between 1/200 – 1/2000 and approximately 1/700- 1/3000 at the male genital tract [5, 98]. However, a number of factors can affect the probability of heterosexual transmission. Presence of genital ulcers and inflammation in either partner [99, 100], high viral load and early-stage disease in the transmitting partner [101-103], sexual risk behavior of one's sexual network [104] and lower socioeconomic status [99, 104] have all been associated with higher risk of transmission. By contrast, couples voluntary counseling and testing (CVCT) in serodiscordant couples [6], early antiretroviral treatment of HIV+ partners in serodiscordant couples [105] and male circumcision [5, 103, 106-110] have been shown to reduce risk of heterosexual HIV-1 transmission. Serodiscordant couples represent those in which one partner is HIV-positive and the other is HIV-negative. Study of these discordant couples has been invaluable in better understanding HIV-1 transmission, evolution, pathogenesis and effective public health interventions.

It is now widely recognized that though chronically infected individuals exhibit a diverse HIV-1 viral quasispecies, the majority of heterosexual transmission events in newly-infected hosts are established by single transmitted/founder (T/F) variants [37, 111]. This finding has prompted investigation and characterization of certain viral characteristics of these T/F viruses that may facilitate their transmission and could potentially be targeted to prevent effective transmission [34, 112-114]. Further analyses of transmitted and non-transmitted viruses in the blood and genital compartments in epidemiologically linked transmission pairs have shown that the T/F virus often is represented as a minor variant in the donor, which again suggests that single-variant transmission involves selection and is not simply stochastic [115]. The severe genetic bottleneck may also be mitigated by factors that affect heterosexual transmission probability overall, including genital ulcers and inflammation, hormonal contraceptives, and presence of other sexually transmitted diseases, leading to a higher probability of multiple variant transmission events (co-infections) [5, 40].

Transmission via percutaneous inoculation (most often associated with injection drug use), MSM and mother-to-child transmission (MTCT), all three of which exhibit higher HIV-1 transmission probability per exposure rates than heterosexual transmission [5, 98], exhibit variable levels of this single-variant transmission bottleneck. While MTCT studies support a strong genetic bottleneck in the context of perinatal transmission of HIV-1 [116, 117], MSM and percutaneous inoculation (via injection drug use) show higher rates of multiple-variant transmission [5, 118].

Historically, genetic and phenotypic properties of these transmitted/founder (T/F) viruses have included the following, though few have been consistently represented across all studies and subtypes: preferential CD4/CCR5-coreceptor tropism [37, 45, 59], shorter variable loops of the Envelope glycoprotein (Env) than donor/chronic variants [40, 111, 119, 120], fewer N-linked glycosylation sites in Env [111,

119, 121], Env sensitivity to transmitting partner's antibodies [111], tier 2 or 3 neutralization sensitivity (meaning that the Envelopes were not particularly easy to neutralize in general) [122] and greater replication efficiency in CD4+ T cells than macrophages in Subtypes A-C viruses [45, 123].

A recent study using Subtype B and C T/F infectious molecular clones (IMC), also suggested higher infectivity, greater incorporation of Env protein, more efficient interaction with dendritic cells and an ability to replicate in CD4+ target cells (even in the presence of Interferon-alpha, which could act early during infection to stimulate an anti-viral innate immune response) [114]. This study compared 27 Subtype B and C T/F IMCs against 12 chronic control IMCs, which were chosen through a phylogenetic clustering strategy, rather than comparing T/F viruses against a matched chronic partner's virus, which is feasible in discordant couple studies alone.

Humoral Responses to HIV-1 Infection

Specificity of anti-HIV-1 antibodies develops over the course of infection and is directly in response to the continually evolving nature of the virus. These antibodies most commonly target the exposed surface of the virion, the Env glycoproteins gp120 and gp41, which mediate viral entry (gp120) and target cell fusion (gp41). Other than antigen-antibody immune complex formation predicted to occur approximately 8 days after detection of viremia [124], non-neutralizing antibodies are generally recognized as the first major anti-Env antibodies to develop post-transmission [38, 87].

Autologous neutralizing antibodies then follow, typically arising within 2-3 months after transmission [38, 46, 125-128] but as early as two weeks post-seroconversion [41]; these effectors have been shown by many groups across multiple HIV-1 subtypes to initially have very narrow neutralization capacities against strain-specific epitopes on autologous transmitted viruses [38, 46, 125-131]. This wave of

monotypic neutralization of the founder virus is frequently followed by viral escape, and this cycle of neutralization and escape occurs repeatedly throughout the time course of infection [125, 126]. Gradually, over the course of 2.5-4+ years post-infection, in about 20% of chronically infected individuals, plasma antibodies capable of neutralizing heterologous HIV-1 viruses develop; this is called heterologous breadth [132-136]. And in a very rare 2-4% of infected individuals, called "elite neutralizers," antibodies capable of potent cross-clade neutralization have been isolated and characterized [137].

HIV-specific neutralizing antibodies (Nabs) bind to cell-free virus via their two antigen-binding fragments, called F(ab), and prevent viral entry into susceptible target cells. Specific regions of the Env trimer commonly elicit early neutralizing antibodies, in a strain-specific manner, including gp41 as well as the variable (V1-V5) and third constant (C3) regions of gp120 [129]. Because of their outward exposure and frequent targeting, the V1-V5 loops have evolved to tolerate substantial sequence variability (via reverse transcriptase-induced mutations) in their escape from antibody pressure [126]. The variable 1 and 2 loop (V1V2) region, although it has been shown to play a role in obscuring or shielding Nab Env epitopes [129, 138, 139], has also been recognized to be a frequent target of autologous neutralizing antibodies itself and subsequent escape from Nabs [42, 128-130, 140, 141]. Changes in V4 and V5 loops have also been observed to mediate effective temporary neutralization escape, suggesting they are also potential targets of autologous Nabs [42, 129, 142, 143]. Antibodies to the V3 loop, a region which participates in determination of CCR5/CXCR4 co-receptor utilization and binding [144, 145], are also commonly seen to be some of the earliest anti-Env antibodies generated, however they are not thought to have a major role in autologous neutralization of primary isolates [129].

Glycosylation of Env trimers (through host glycosylation machinery) is another immune evasion mechanism of HIV-1 that obscures the otherwise exposed outer domain

Nab targets of native Env through steric hindrance of bulky carbohydrates; this simultaneously creates an immunologically silent face on the trimer because of the host-derived, non-immunogenic nature of the sugars [146, 147]. Changes in these carbohydrate moieties through addition, deletion, and shifting of potential N-linked glycosylation sites constitute effective mechanisms of neutralization escape that do not disturb functional regions of gp120 such as the receptor binding site [125].

Vaccinologists have historically striven for neutralizing antibody elicitation because all successful FDA-approved vaccines to date have conferred protection at some capacity through neutralizing antibody responses [87, 148]. Specific to efforts against HIV-1, evidence exists that Nabs, polyclonal HIV immunoglobulin and broadly cross-reactive monoclonal antibodies administered via passive immunization in non-human primate and mouse models provide protection against viral challenge [82-91, 94-96].

Non-neutralizing antibodies, also bind viral antigen yet, by contrast, cannot prevent viral entry. These binding antibodies exert anti-viral activity by increasing mucosal barrier protection, sequestering virus at the genital tract and epithelium, and facilitating effector activity through the binding interaction of their crystallizable fragment (called F(c)) with innate immune cells expressing Fc-receptors (e.g. natural killer cells, monocytes, macrophages, dendritic cells, neutrophils, etc.) [87, 149-151]. Specifically, virus-specific antibody molecules that simultaneously bind both viral antigen on the outside of infected cells (via F(ab)) and Fc-receptors on immune cells (via F(c)) can participate in a process called antibody-dependent cellular cytotoxicity (ADCC), in which these recruited innate effector cells initiate a degranulation cascade which results in virally-infected target cell death via apoptosis [150].

Certain studies have suggested that non-neutralizing antibodies target Env residues that are exposed during gp120 shedding, non-native or non-functional Env structures, or viral debris from infected cells and could detrimentally divert attention

away from more effective neutralizing targets [146, 147, 152, 153]. Recent immunological correlates analyses from the RV144 vaccine efficacy trial [92], however, have attributed the moderate protection seen in uninfected vaccinees to the presence of specific binding (but non-neutralizing) antibody responses to Env [93]. These somewhat surprising immunological correlates of risk and protection have been the source of extensive follow-up investigations [93, 154, 155]. Higher levels of binding antibodies to V1V2 were associated with protection in vaccinees that remained uninfected, while high gp120 constant region 1 (C1)-specific plasma IgA was associated with risk of infection and potential mitigation of IgG-mediated antiviral activity via ADCC [93, 154, 155]. Therefore, the active role of non-neutralizing responses in protection of HIV-1 infection has recently merited more thorough investigation.

Identification of HIV-1 Superinfection

HIV superinfection has been defined as a re-infection with a heterologous HIV-1 variant after an HIV-infected individual has already had an opportunity to mount an immune response to the primary infection [156]. Preliminary evidence for HIV superinfection was found in the mid-1980s, initially through observations of HIV-1/HIV-2 dual infections [157, 158] and also in experimentally-induced HIV superinfection in chimpanzees [159]. Further evidence of HIV-1/HIV-2 superinfection [160] and intergroup HIV-1 Group M and O superinfections [161] were also later described, though these cases appear to be rare and more anecdotal, and the focus of this section will be on identification of HIV-1 (intragroup M) superinfections.

Despite suggestive evidence of the potential for superinfection, it was not until 2002 that 4 documented clinical cases of HIV-1 superinfection in high-risk individuals surfaced nearly simultaneously [162-164]. Ramos et al. described two injection drug users from Thailand who showed evidence of primary infection by either a CRF01_AE

recombinant with subsequent superinfection by Subtype B variant, or vice versa. Jost et al. and Altfeld et al. subsequently published on single MSM cases that acquired intersubtype [163] or intrasubtype [162] superinfection during treatment interruption, concomitant with viral load spikes at the time of superinfection. In these cases, confirmatory restriction-fragment length polymorphism (RFLP), sequence- or subtype-specific polymerase chain reaction (PCR) methods were used to confirm the presence of distinct viral populations before and after the putative superinfection [162-164]. Unfortunately, this gave the first indication that primary immune responses, in these HIV+ individuals, were insufficient at protecting against HIV-1 reinfection; a lesson of great importance when considering cross-protective vaccine feasibility [55, 156].

Superinfection detection methods evolved over the course of the next 10 years, relying more heavily on viral sequence-based approaches, including viral population (or bulk) sequencing, single-genome amplification of individual viral variants and clonal sequencing, rather than previous techniques with relatively limited detection capacities (including heteroduplex mobility assays, multiregion hybridization assays and RFLP analysis) [55, 156]. Recently, high throughput 454 deep sequencing of viral regions has also further advanced the limit of detection for identifying superinfections and is especially useful in detecting minor superinfecting variants [55, 165-167].

Major remaining limitations in the identification and interpretation of superinfection cases are the limited numbers of enrolled patients in these studies and availability to only cross-sectionally collected samples (rather than longitudinal samples), which does not allow for successful interpretation of timing of the secondary infection and ability to distinguish between co-infection and superinfection [168]. However, with the increased sensitivity of sequence-based approaches, and a growing interest in population-based studies looking at incidence of superinfection, superinfection cases have since been widely recognized to occur across the world and at

variable frequencies and rates, sometimes as high as primary infection in certain cohorts [55]. Many studies identified superinfections occurring in high-risk cohorts such as commercial sex workers (CSW) or female bar workers [169-173], injection drug users [48, 164] or MSM [162, 163, 174-177], likely owing to the fact that the probability of HIV-1 transmission is higher in these groups as compared to heterosexual exposure [5]. However, more recently, superinfections have also been shown to occur in heterosexually infected seroconverters from seroconcordant couple cohorts [50, 55, 166, 167].

Risk factors of superinfection have not clearly been defined, as large-scale population studies of substantial enough numbers of superinfections and matched controls have not yet been coalesced. However, common risk factors associated with primary HIV-1 transmission are thought to also affect risk of superinfection including potential exposure to virus through unprotected sexual activity, more sexual partners, non-spousal partnerships, genital infections and inflammation, shared needle use, as well as regional HIV prevalence [55, 168]. Encouragingly, though, behavioral studies have shown that if HIV+ individuals are counseled about the risk of superinfection and the implications it could have on their own health, they were more likely to report safer sexual practices, which supports the idea that awareness of superinfection can affect sexual risk behavior [178].

Although a few groups have observed no evidence of superinfection in population studies of treated individuals and high-risk MSM individuals [179, 180], rates of superinfection in most population-based studies appear to generally parallel trends of primary transmission, but again can be affected by a multitude of logistical, sociodemographic, behavioral and clinical factors (including antiretroviral drug use, longitudinal follow-up length, screening methods, etc.) [55, 168]. In a recent study of heterosexual seroconverters in rural Uganda, incidence of superinfection was compared to rate of primary infection in 20,200 initially uninfected individuals in the area after

controlling for risk factors affecting either group; here, the rates of superinfection and primary infection were similar (1.4 per 100 person years vs. 3.28 per 100 person years) [167].

Immune Responses in HIV-1 Superinfected Individuals

The majority of reported superinfection cases, with longitudinal follow-up from close to the time of primary infection, have observed superinfections occurring during early infection and within or around the first year of infection [50, 164, 169, 177, 181, 182]. Mathematical modeling in a subtype B study has also showed a dramatic 21-fold reduction in risk of superinfection after the first year of infection [183]. Similarly, a study of 31 Subtype C female sex workers identified 6 women to be dually-infected within 3 months of infection, however what type of dual infection this was (co-infection vs. superinfection within 3-months of primary infection) could not be determined; therefore, these individuals were simply recognized to have high risk of dual infection during early infection [184]. Interestingly, in the remaining women recognized to initially have a single homogeneous viral population at time of enrollment in this study, there was no further evidence of superinfection during 24 months of follow-up [184]. These studies in combination suggested the hypothesis that superinfection (or dual infection) risk may be greatest during this window due to the lack of a developed and potentially cross-protective immune response during early infection and that risk of superinfection may wane over time [55].

This hypothesis has been supported by immunological studies showing weak autologous neutralizing antibodies [162, 177, 185], heterologous or cross-protective antibodies to lab-adapted strains [177] prior to superinfection or potentially lower cross-reactive CTL responses in superinfected individuals [186]. However, identifying immune correlates of risk of superinfection has been difficult and somewhat controversial due to

the small numbers of identified superinfection cases, inherent differences in study participants (including subtype of viruses infected, behavioral characteristics, transmission risk and likely exposure to virus, etc.), availability of samples to answer immunological questions, lack of early post-transmission samples and subsequent longitudinal follow-up and basic differences in how studies were executed [55]. Therefore, only the main studies suggesting or refuting immunological deficits in superinfected individuals will be addressed here.

Smith et al. published a study of 3 MSM intrasubtype B superinfected individuals superinfected during the first year of infection and compared autologous and heterologous neutralization in these individuals against 11 non-superinfected controls from the same cohort. In this study, immune responses in only two time points were evaluated, called baseline (at the first sample date, and prior to superinfection) and a 6-month time point (representing post-superinfection). Timing of superinfection was not narrowly defined within this time frame, and the authors did not clearly state how autologous virus from baseline and superinfecting virus from 6-month visits were isolated. The authors acknowledge that the first sample dates in the superinfected group tended to be later than controls, raising concerns of whether the autologous virus is representative of an early transmitted-founder-like population or a subsequent early escape variant.

Using plasma from baseline, these authors showed that superinfected individuals exhibited low autologous neutralizing titers to contemporaneous baseline virus, and lower heterologous neutralization of two lab-adapted HIV-1 strains (JR-CSF and NL4-3) compared to the controls. Measuring heterologous neutralization to lab-adapted strains is not the same as measuring breadth to a panel of Envs derived from primary isolates and lacks clinical relevance. Importantly, though heterologous neutralization of lab-adapted strains prior to superinfection was evaluated, cross-neutralization of the

superinfecting variant prior to superinfection was not. Still, this study gave the first evidence that there may be a humoral antibody deficit in superinfected individuals that could contribute towards predisposition to superinfection [177].

In a follow-up study of the two initially characterized intersubtype superinfected cases (Ramos et al.), the authors also investigated CTL responses in singly-infected injection drug users in the same cohort (infected with either Subtype B or CRF01-AE variants) and retrospectively compared these responses to those in the two superinfected cases [164, 186]. The authors found there to be higher levels of cross-reactive CTLs to peptides of the superinfecting subtype in the newly evaluated singly-infected controls, however direct comparisons of cross-protective CTL responses between the superinfected and singly-infected groups in the same study were not performed, and therefore this difference may be somewhat speculative [186].

Despite studies linking superinfection with early immunological deficits and a propensity towards early superinfections based on case reports and population studies, researchers have also found evidence of superinfections during chronic infection, anywhere from 2 to 12 years post-infection [48, 162, 163, 170, 171, 187]. This indicates that superinfections may not exclusively occur during this early window post-infection when anti-HIV immune responses may be underdeveloped. In fact, superinfection events after the establishment of broad CTL responses and neutralizing antibodies have also been observed and suggest that in these settings [162, 171], the elicited immune responses were not protective.

Altfeld et al. reported that in one chronically-infected Subtype B MSM individual, who was superinfected during treatment interruption, broad CTL responses capable of targeting multiple epitopes in the primary infecting virus were unable to provide functional protection against intrasubtype superinfection [162]. Similarly, Blish et al. showed in a case-controlled study evaluating the magnitude and polyfunctionality of

CD4+ and CD8+ T lymphocytes (following stimulation with global HIV-1 peptide pools) prior to superinfection in 10 inter and intrasubtype superinfected Kenyan CSW and at similar time points after primary infection in 28 non-superinfected matched controls, that there was no association between cellular immune responses and the odds of superinfection [188]. Because of the viral dynamics following superinfection, which can often lead to complete replacement of the primary infecting virus by the superinfecting virus, even broad CTL targeting to multiple epitopes in the primary infecting virus may not confer cross-protective viral control of a superinfecting variant due to viral sequence variation which may confer CTL escape. In such cases, viral escape through superinfection and potential subsequent recombination may result in loss of immune control [52, 162, 189].

In contrast to the work by Smith et al., showing low neutralizing antibodies to both autologous and heterologous viruses prior to superinfection, research from a well-characterized cohort of Kenyan CSW (Blish et al.) has shown that there did not appear to be a difference in cross-protective neutralizing antibodies prior to superinfection between 6 superinfected and 18 case-matched non-superinfected controls [171]. It is important to note a few important differences in this study. In this mixed clade cohort (viruses of Subtype A, C, D and various recombinants are common), half of the identified superinfections were intrasubtype A and the other were mixed intersubtype superinfections. Also, superinfections were observed over a wider time scale, 2/6 occurring within the first year and 4/6 occurring after 2 years post-infection.

The authors were primarily interested in looking at differences in cross-protective or heterologous neutralizing antibody breadth to a much larger and clinically relevant panel of pseudoviruses representing transmitted Envs from across multiple subtypes, rather than autologous neutralization of early variants. However, in 3/6 superinfected cases, autologous neutralization of “initial” variants were tested

longitudinally, starting from the time point directly prior to superinfection. These "initial" variants were isolated and cloned from the time point after superinfection, and as some of these superinfections occurred during chronic infection, these variants cannot reasonably be called "initial" variants when cloned from such chronic time points, as they likely have evolved significantly over 2+ years of infection. Therefore it is hard to reconcile and directly compare the two studies and their findings. However, this study was the first to suggest that, in this cohort, there were no significant cross-protective neutralizing antibody deficits prior to superinfection [171]. It is of interest to note that while there were no differences in heterologous breadth prior to superinfection, the authors did observe lower neutralization breadth and potency at one year post-infection in superinfected individuals compared to their case-matched controls. This could suggest that while there may have been a difference in the humoral response during early infection, other factors may have affected susceptibility to superinfection in these individuals.

These conflicting studies, which could potentially be very important in guiding our understanding of why natural infection fails to prevent re-infection in these individuals, are underpowered by the number of superinfection cases, and researchers have recently addressed this problem by suggesting collaboration in order to strengthen the power of these immunological studies into discernable meta-data analysis [55]. Despite conflicts about immune responses prior to superinfection, studies of post-superinfection immune responses seem to generally report a boosting of neutralizing antibody titers after superinfection [185, 190, 191], which may also be of importance in the context of vaccination regimens.

Clinical Implications of HIV-1 Superinfection

Similar to the issues of characterizing immune responses to superinfection, understanding clinical implications and outcomes of superinfection has been difficult due to availability of samples to evaluate these clinical outcomes, longitudinal follow-up and numbers of superinfections characterized. Therefore, although several case reports have evaluated various aspects of clinical progression after dual infection, including increasing viral load set-point [184], accelerating disease progression towards clinical AIDS end points [174, 176, 181] and loss of immune control [52, 162], most of these studies must be considered anecdotal due to the fact that they report, in most cases, single superinfected patients. Superinfection has also rarely been reported to occur in either elite viral controllers or long-term non-progressive patients with no immediate clinical manifestations [192, 193].

A few studies have consistently observed similar clinical trends, including: increased viral load spike at the time of superinfection [162, 163, 176, 181, 194], frequent and rapid viral recombination post-superinfection [47, 49, 50, 52, 195] and transmission or masking of drug-resistant HIV-1 strains [48, 175, 194, 196, 197]. All of these factors can have potentially adverse effects on clinical disease progression and efficacy of treatment regimens, therefore understanding on a larger-scale which effects are real and which are more anecdotal is important for treatment of superinfected patients, especially as the increasing presence of recombinant viruses and population studies suggest that superinfection occurs fairly frequently worldwide.

Summary

Understanding if and why, on an immunological basis, some individuals become HIV-1 superinfected while others, who may be similarly exposed to exogenous HIV-1 virus, remain only singly infected is critical to supplementing our understanding of what vaccine-mediated immune responses could confer protection from primary HIV-1 infection. Thus, this body of work first focuses on identification and characterization of superinfected individuals within the clinically relevant context of low-risk Subtype C-infected heterosexual couples, of which most new primary infection events occur [6].

In chapter two of this manuscript, we describe the combination of detection methods used to screen twenty-two epidemiologically unlinked transmission pairs in a Zambian cohort of heterosexual couples, in which both partners were longitudinally followed by viral sequencing and phylogenetic analysis, for evidence of superinfection. Here too, as in previous studies of high-risk individuals [133, 164, 170, 171, 176, 177, 182], superinfection was found to occur with high frequency, as intrasubtype C superinfection (from outside partnerships) was detected in 3/22 acute partners. Rapid viral recombination was also observed post-superinfection, consistent with other studies [47, 49, 50, 52, 195], though the functional implications of these recombination events were not evaluated.

In chapter three, we delve deeper into the primary immune responses in the three identified intrasubtype C superinfected individuals. The fact that superinfections were found to occur within the first year, consistent with cases from other studies [50, 164, 169, 177, 181-183], suggested that poor early immune responses may predispose individuals towards superinfection [177]. We systematically tested for humoral antibody deficits prior to superinfection by comparing multiple facets of the anti-HIV antibody response in superinfected individuals against those of 10 non-superinfected matched controls from the same study.

Specifically, we examined autologous neutralizing antibodies to the founder virus over the first year of infection, Env-specific binding antibodies and the potential for heterologous neutralizing antibody breadth to both superinfecting variants as well as an established subtype C panel of pseudoviruses [198, 199]. Data from this body of work suggests that poor early immune responses (as reflected by autologous neutralizing antibodies to founder virus and Env-specific binding antibodies) may predispose individuals towards superinfection; and, if confirmed on a much larger scale, these results could support the feasibility of intrasubtype protection from primary HIV-1 infection via an HIV-1 vaccine.

Chapter 2

Timing and source of subtype-C HIV-1 superinfection in the newly infected partner of Zambian couples with disparate viruses

Published in *Retrovirology,* 2012, 9:22.

Colleen S. Kraft and Paulina A. Hawkins performed the *env* single genome amplification of superinfected individuals (Figures 2-4, 5A), while Debby Basu performed the screen of the 22 transmission pairs for superinfection via gp41 population sequencing and degenerate base counting (Figure 1, 5B). Colleen Kraft and Debby Basu performed the recombination analysis (Figure 6).

Colleen S. Kraft, Debby Basu and Eric Hunter wrote the manuscript.

Abstract

Background

HIV-1 superinfection occurs at varying frequencies in different at risk populations. Though seroincidence is decreased, in the negative partner of HIV-discordant couples after joint testing and counseling in the Zambia Emory HIV Research Project (ZEHRP) cohort, the annual infection rate remains relatively high at 7-8%. Based on sequencing within the gp41 region of each partner's virus, 24% of new infections between 2004 and 2008 were the result of transmission from a non-spousal partner. Since these seroconvertors and their spouses have disparate epidemiologically-unlinked viruses, there is a risk of superinfection within the marriage. We have, therefore, investigated the incidence and viral origin of superinfection in these couples.

Results

Superinfection was detected by heteroduplex mobility assay (HMA), degenerate base counting of the gp41 sequence, or by phylogenetic analysis of the longitudinal sequences. It was confirmed by full-length *env* single genome amplification and phylogenetic analysis. In 22 couples (44 individuals), followed for up to five years, three of the newly infected (initially HIV uninfected) partners became superinfected. In each case superinfection occurred during the first 12 months following initial infection of the negative partner, and in each case the superinfecting virus was derived from a non-spousal partner. In addition, one probable case of intra-couple HIV-1 superinfection was observed in a chronically infected partner at the time of his seroconverting spouse's initial viremia. Extensive recombination within the *env* gene was observed following superinfection.

Conclusions

In this subtype-C discordant couple cohort, superinfection, during the first year after HIV-1 infection of the previously negative partner, occurred at a rate similar to primary infection (13.6% [95% CI 5.2–34.8] vs 7.8% [7.1–8.6]). While limited intra-couple superinfection may in part reflect continued condom usage within couples, this and our lack of detecting newly superinfected individuals after one year of primary infection raise the possibility that immunological resistance to intra-subtype superinfection may develop over time in subtype C infected individuals.

Background

HIV-1 superinfection presents an additional concern to the already challenging problem of HIV-1 vaccine design in the face of the virus's rapid evolution [1]. Superinfection is defined as a reinfection by a heterologous HIV-1 strain after a primary immune response has already been mounted [2]. Superinfection and coinfection (primary infection with two genetically distinct viruses) differ based on whether the second infection is contracted prior to or after the host immune response has been mounted [3]. The first documented case of superinfection was identified in a high-risk MSM individual, initially infected with a CRF01_AE subtype followed by a subtype B superinfection after two years [4]. Several other cases have been reported, demonstrating a spectrum of intersubtype [5-12], intergroup [13] and intrasubtype [14-17] superinfections.

Many studies have raised questions about the frequency of superinfection and were unable to identify HIV-1 superinfection in the populations under investigation [18-21]. Despite these doubts, HIV-1 superinfection has now been seen to occur at frequencies comparable to primary infection in certain cohorts [5,17]. The behavioral aspects of these cohorts impact transmission [22] and the interplay between the risk for re-exposure [23], as well as the regional HIV-1 prevalence have been thought to influence the likelihood of HIV-1 superinfection in a given population [3].

It is known that HIV-1 superinfection occurs despite broad CD8+ T-cell [14] and cross-reacting neutralizing antibody responses [24], although it appears that there is less likelihood for HIV-1 superinfection later in the course of HIV infection [17,25,26]. Studies have evaluated the neutralizing antibody population around the time of

superinfection and demonstrated both lack of neutralizing antibody [27] as well as robust neutralizing responses [24].

HIV-1 superinfection has clinical ramifications. Transmission of drug resistant variants through superinfection has been well described [16,17,28-30] and there has been evidence of increased viral load set-points in individuals who are dually infected [31,32] or superinfected [14,16]. The numerous circulating recombinant forms of HIV-1 demonstrate that dual infection of individuals [9,33,34] and the resulting superinfection can contribute to the overall diversity of a virus population. Modeling has shown that intrasubtype superinfection may be as high as 15% in some populations based on evidence of recombination [35], and superinfection followed by recombination may contribute to immune escape within an individual [36].

Since 1994, the Zambia Emory HIV Research Project has followed a cohort of HIV-1 discordant couples, where one partner is HIV-infected and the other is HIV-uninfected. Joint counseling and condom provision in such couples can reduce transmission significantly [37-40]. When HIV-1 infections occur, approximately one in eight are acquired from non-spousal partners, leading to a couple infected with genetically distinct viruses [41]. We have followed 22 of these epidemiologically unlinked couples longitudinally for at least 1 year and up to 5 years, to determine the frequency and nature of superinfection in this cohabiting heterosexual population. We observed superinfection in four out of 44 individuals, but only one of these involved transmission of virus from one spouse to the other. The other superinfections resulted from transmissions from non-spousal partners within one year following a primary HIV-1 infection acquired in an extra-marital relationship. Thus, superinfection from non-spousal partners occurs more commonly than between spousal partners in this cohort,

although evidence for continued condom use between spousal partners could limit the incidence of intra-couple HIV-1 superinfection.

Materials and Methods

Zambian cohort

The Zambia Emory HIV Research Project (ZEHRP), a Rwanda Zambia HIV Research Group (RZHRG) site in Lusaka, Zambia, was established in order to study heterosexual cohabiting HIV-1 discordant couples, and provides voluntary testing and counseling as well as long-term monitoring and health care to participating couples [54,55]. HIV discordant couple is defined in this cohort as a couple that upon screening and enrollment has one HIV-infected partner (seropositive index partner) and one HIV-uninfected partner [56]. This screening is based on rapid HIV-1 antibody test positivity [54,57]. Both partners are followed quarterly with repeat counseling and documentation of reported sexual exposures within and outside the marriage, and assessment of biological markers of unprotected sex [37]. Plasma from the seronegative partner is tested at every visit for HIV-1 antibodies with rapid tests, and for the presence of p24 antigen using the Vironostika® HIV-1 p24 antigen ELISA [54,57]. Despite counseling and provision of condoms, and a two-thirds reduction in transmission [58], approximately 7%-8% per year of the initially seronegative partners are infected by HIV-1. Once a transmission event had been established, the newly infected partner was followed quarterly, and the chronically infected partner at least annually. Blood products (PBMC and plasma) were collected at each visit under protocols approved by the University of Zambia Research Ethics Committee and the Emory Institutional Review Board. Plasma was obtained by centrifugation of whole blood, and stored in aliquots at −80°C until use. Viral RNA was extracted from these samples using the QIAamp® Viral RNA Mini kit (Qiagen Inc., Valencia, CA). Individuals who meet criteria for antiretroviral therapy are referred elsewhere and drop out of the cohort studies.

Viral gp41 sequences from newly infected, previously seronegative, individuals and their chronically infected partners were used to define epidemiologic linkage of the transmission as described by Trask et al., 2001 [41]. During the period 01/01/2002 to 06/01/2007, a total of 202 seroconversions were identified, of which 49 (24%) were classified as unlinked. A total of 22 couples were selected for further study based on the criteria: 1. Samples corresponding to at least one year of follow-up were available at Emory University, and 2. The seropositive index partner had a viral load greater than 1000, because we were interested in determining the frequency of superinfection within the couple and primary transmission from such individuals is rare. A table detailing the available demographic, clinical and behavioral characteristics of these individuals is provided in Additional file 1: Table S1. Although the exact time of transmission is not available, for each newly infected partner, samples were collected within a median of 91 days (range 10–181 days) of the last seronegative visit. For this study, we have defined two different types of dual infection: co-infection is defined as the detection of two genetically distinct viruses at the time of seroconversion in the previously HIV-1 negative partner; superinfection is defined as the detection of more than one genetically distinct virus at least 3 months after primary infection seroconversion in the seronegative partner. For the seropositive index partner, superinfection was defined as the detection of a novel genetically distinct variant at or after the time of infection of their seronegative partner.

HIV-1 gp41 and gag nested PCR

Viral RNA was reverse-transcribed using SuperScript® III One-Step RT-PCR System with Platinum® Taq High Fidelity as per manufacturer's guidelines (Invitrogen Co., Carlsbad, CA). Nested PCR amplifications using Expand High Fidelity polymerase (Roche Applied Science, Indianapolis, IN) were performed for gp41 as previously

described and in Additional file 2: Methods [41]. Purified positive amplicons were sent out for direct sequencing to MWG Sequencing (Huntsville, AL). Nested PCR for gag was accomplished using the following primers: Outer: 5′ – TTC TAC GGA GAC TCC ATG ACC C – 3′, 5′ – ATT TGA CTA GCG GAG GCT AGA A – 3′, Inner: 5′ - ATT GCT TCA GCC AAA ACT CTT GC - 3′, 5′ - CGA CCA AAA TTA CCC TAT AGT GCA G - 3′, and sequencing primers: 5′ - GGG ACA TCA AGC AGC CAT- 3′, 5′ - GCC AAA GAG TGA TTT GAG GG - 3′.

Sequence analysis and highlighter analysis

Sequences were analyzed from amplicons in Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI). Geneious Pro (Biomatters Ltd, Auckland, New Zealand) software was used to align sequences, and neighbor-joining trees were generated using the Tamura-Nei genetic distance model with the bootstrap resampling method. Additionally, the Highlighter tool from Los Alamos National Laboratory HIV Sequence Database (http://www.hiv.lanl.gov/content/hiv-db/HIGH-LIGHT/highlighter.html) was used to map mutations deviating from the earliest sample. APOBEC G to A mutations (open diamonds) and degenerate bases (Dark Blue) were quantified in a longitudinal fashion within the acute transmission partner's virus with respect to the viral sequence from the time of seroconversion. Recombination analysis was performed using the Highlighter tool for analysis of the presumed parent and daughter sequences.

Env single genome analysis (SGA)

Single genome PCR amplification was performed of the entire *env* gene [42,43]. Single genome analysis was conducted on couples who were determined to have dual infection by the screening methods of degenerate base counting, HMA or phylogenetic analysis of

sequences encoding gp41. Full-length *env* gene sequences were analyzed for superinfection cases (ZM211M, ZM211F, ZM282M, ZM247F).

Degenerate base counting

After obtaining the sequences from gp41 PCR amplicons, degenerate or ambiguous codes were manually counted using the International Union of Pure and Applied Chemistry (IUPAC) designations. Only nucleotide positions where the secondary (and occasionally tertiary) peak was at least 30% as high as the primary peak was counted as a mixed peak, and these had to be present in both forward and reverse primer sequences. Degenerate codes were then assigned to the mixed nucleotide accordingly.

Heteroduplex mobility assay

Second round gp41 PCR products amplified from plasma were used directly in the heteroduplex assay as described elsewhere [8,59].

HIV-1 quantitative viral loads

HIV-1 viral load determination was performed on plasma using the Amplicor HIV-1 Monitor Test, v 1.5 (Roche Diagnostics, Indianapolis IN).

Statistical analysis of behavioral data

A univariate analysis to compare the superinfected and non-superinfected groups was performed using the Wilcoxon rank sum test, or Fisher's exact test as appropriate, for continuous variables. Categorical variables were compared using the chi-square statistic. All analyses were performed using SAS® version 9.2 (Cary, NC), and p-values <0.05 were considered to be statistically significant. The 95% confidence interval for incidence of infection was calculated based on the method by Clopper and Pearson [60].

Results

Selection of the study couples

Two hundred and two HIV-1 discordant couples who seroconverted to concordant infected status (both partners HIV-infected) from 2002–2008 in the ZEHRP cohort were screened for epidemiologic linkage as described previously [41]. In this subset of 202 couples, 49 (24%) were found to have partners with genetically distinct viruses (epidemiologically unlinked transmissions), and 22, selected as described in Methods, were screened for HIV-1 superinfection. Three approaches were employed (see Methods): 1) quantitation of degenerate bases in viral population sequences of the genomic regions encoding the ectodomain of gp41 and gag, 2) phylogenetic tree and Highlighter tool analyses of these sequences, and 3) heteroduplex mobility assay (HMA) of gp41 amplicons. If any of these methods suggested dual infection (either superinfection or co-infection – see Methods), longitudinal single genome amplification (SGA) of the full-length *env* gene was performed in order to further confirm and characterize the dual infection. Of the 22 acutely infected individuals, there were 9 women and 13 men; none of the participants reported undergoing antiretroviral therapy or engaging in risk behaviors other than heterosexual sex. The ZEHRP cohort is primarily (96.9%) subtype C [41,42], and as expected, all 44 of the individuals had primary infection with subtype C HIV-1. The length of screening for individuals ranged from 12–66 months, with at least 3 time points analyzed during the length of the screening for the acute partner.

Identification of dual infections using PCR amplified gp41 sequences and highlighter tool analysis

For each individual, a 399 bp fragment within the gp41 ectodomain region of the *env* gene was PCR amplified from each longitudinal sample time point as described in Methods. Degenerate bases (DB) were scored when a secondary peak exceeded 30% of the major peak height in the sequence traces. A comparison of the maximum number of degenerate bases at any time point (as a percentage of the 399-bp gp41 ectodomain sequenced) and the maximum pairwise distance (PD) between the month 0 virus sequence and that of the most divergent viral sequence was performed (Figure 1A, B). For the acutely infected individuals this analysis revealed two distinct groups of individuals (Figure 1A). A majority of the individuals clustered in the low percentages (<4% maximum PD, <3% maximum DB), while four exhibited high percentages of both parameters (>7% maximum PD, >6% maximum DB). One of these (ZM215F; arrowhead) had been shown previously to be a case of co-infection with two genetically distinct variants from a single donor differing by a PD of more than 9% [42,43], while additional phylogenetic analyses (described below) identified the remaining three individuals (black arrows) as cases of superinfection (ZM282M, ZM211F, and ZM247F).

A representative Highlighter plot (www.hiv.lanl.gov) was compiled for each individual's population sequences and provided a visual representation of homogeneity or heterogeneity of the viral population at each time point relative to the virus population at the time of the acutely infected partner's seroconversion (month 0) (Figure 1C-F). Nucleotide changes from the month 0 sequence are demonstrated with tick marks that bear colors unique to each nucleotide (A = green, T = red, G = orange, C = light blue, Degenerate/ambiguous = dark blue).

This analysis showed that for a majority of the acutely infected individuals (18/22), the gp41 sequence remained relatively homogeneous with no evidence of dual infection. An example of this is shown in Figure 1C for subject ZM289M, who exhibits only minimal changes (1 base change at 3 months, 12 months, 24 months, and then 2 changes at 30 months) in the gp41 sequence of the infecting HIV-1 strain over 30 months. However, in the three individuals identified above evidence of superinfection was obtained (Figure 1D-F). In addition, for one individual, ZM215F, evidence for co-infection by distinct variants from a single donor was observed, confirming a previous study [43].

ZM282M (Figure 1D) has few base changes until 10 months, at which point there are 49 degenerate base changes observed, consistent with a mixture of genetically distinct viruses at this time-point. Interestingly, the Highlighter plot shows that the superinfecting virus present at 10 months persists until 36 months when there was evidence for emergence of a dominant recombinant virus (see below). This superinfecting virus predominance is evident from the resolution of mixed bases (blue ticks, representing mixed bases) to simple mismatched bases that are derived from the superinfecting strain compared to the month 0 viral sequence.

Individual ZM247F (Figure 1E) was previously reported to be co-infected by closely related variants (PD 2.7%, corresponding to 11 nucleotide differences in gp41) from the same individual at the time of acute infection [43]. At month 3 post-infection there is evidence of superinfection by a genetically distinct virus (PD ~12%) that at this time point becomes the predominant strain, then at 18 months and 21 months, a significant number of degenerate bases are observed (40 and 36, respectively) consistent with a re-emergence of the initial virus strain that results in a mixture of it and the

superinfecting virus in the plasma. At 24 months, a recombinant of the superinfecting strain again starts to dominate.

ZM211F (Figure 1F) resembles ZM282M in that the virus sequence is homogeneous until month 9 where there is clear evidence for superinfection, with a mixture in the viral population as seen by degenerate bases. By month 18, the superinfecting virus sequence has become dominant and remains the predominant strain until at least 33 months.

Thus through a combination of degenerate base and phylogenetic analyses on longitudinal sequenced samples, we identified three cases of superinfection in this cohort. In order to rule out the possibility that we might have missed cases of superinfection because of rapid recombination between the superinfecting virus and the initial infecting variant [44], we performed the same analysis on a 400 bp segment of the *gag* gene encoding a region of p24. The results of this analysis (data not shown) did not reveal any additional cases of superinfection.

Clinical characteristics of superinfected individuals

Table 1 shows the sexual behavior data collected from self-reported questionnaires for the 22 acutely infected individuals abstracted from 2002–2010. The three acutely infected individuals that were superinfected (ZM282M, ZM247F, ZM211F) are compared against the 19 acutely infected individuals that were not superinfected. The comparison between groups was limited to the first 12 months of primary infection during which initial superinfection was observed. The ages were similar between the two groups. Genital infections or ulcers were reported or visualized in all three participants

in the superinfected group, and in 36% of the not superinfected group, which was statistically significant (p = 0.01). Two individuals in each group had a positive RPR titer in the first year of their HIV-1 infection (p = 0.02), and there was no difference between *Trichomonas* infection within the couple between the groups (p = 0.31). All individuals in the superinfected group had sexual intercourse with at least one partner between each visit (total of 12 visits over 1 year for 3 individuals). The superinfected group reported 201 episodes of sexual intercourse with condoms and 29 (12.6% of total) episodes of sexual intercourse without condoms (mean values are shown in Table 1). Sex with a non-spousal partner (11 episodes) was reported by one individual, ZM282M, while both superinfected women denied extra-marital contact. The non-superinfected group reported 2042 episodes of sexual intercourse with condoms, and 104 episodes (4.8%) without condoms. Three individuals in the not superinfected group reported having extra-marital partners (ZM249M, ZM250M, ZM184F). Only 1 of the newly infected women became pregnant, although 4 of the cohabiting female partners of the 13 acutely infected men became pregnant. All men in this study were uncircumcised.

Analysis of incidence

The incidence of superinfection was determined over 12 month periods after seroconversion, and this was compared against the calculated incidence of primary transmission within the larger cohort of enrolled sero-discordant couples. The first 12 months showed 22 couples with an incidence of superinfection of 13.6 (5.2–34.8, 95% CL) per 100 person years (py). During months 12–24 and 24–36, there were no further cases of superinfection in the remaining 19 patients.

All person-years of observation in the prospective study were used to calculate overall HIV-1 incidence rates in the broader cohort. Seroconversion and transmission rates were calculated including all seroconversions. Exact distribution methods were used to calculate 95% confidence intervals. In the first 3 months, the rate was 13.1 (10.6–16.1, 95% CL), from 3–12 months after enrollment, the rate was 7.9 (6.5–9.4, 95% CL), and the rate between 12–24 and 24–36 months was 7.4 (5.9–9.0, 95% CL) and 7.2 (5.4–9.3, 95% CL), respectively. The higher incidence of infection observed during the first 3 months following enrollment likely reflects infections acquired immediately prior to couples counseling and condom provision and that were still in the antibody negative phase at the time of enrollment.

A comparison of incidence of superinfection in the recent seroconvertors during the first year (13.6/100py) to the incidence of primary infection (per 100 person years) in the broader discordant couple cohort during either the 0–3 month (13.1) or 3–12 month (7.9) periods using the t-test assuming equal variance yielded no statistically significant differences.

Characterization of superinfection by single genome amplification

In order to better understand the dynamics of superinfection in the three individuals identified, single genome amplification (SGA) of full-length *env* gene was performed. The neighbor-joining (N-J) phylogenetic tree of the sequences obtained for the male and female in couple ZM282 is shown in Figure 2. Sequences from the chronically infected partner, subject ZM282F (black), cluster distinctly from the male's sequences (blue), confirming that these individuals are an unlinked transmission pair, and her sequences exhibit up to 3.5% diversity (pairwise distance) consistent with that of

a chronically infected individual. For the male, the nearly identical sequences from the earliest time point (M_0) branch together, consistent with a genetic bottleneck in which a single genetic variant was transmitted [42,45,46]. Limited genetic heterogeneity was observed over the next 8 months with the *env* sequences differing by only 1.1% over this time. By contrast, at the M_10 time point, two distinct virus populations were detected, with approximately 1/3 of the sequences forming a distinct, genetically distant branch that is approximately 12.5% divergent from the initial infecting virus (red). This is consistent with the time of superinfection observed from population sequence analysis of the gp41 encoding region (Figure 1D). At subsequent times (12 and 18 month) there are sequences present that cluster with the superinfecting virus and others that form another distinct branch that represent recombinants (see below). Only a minority of the sequences from the later time points cluster with the initial infecting virus population, consistent with the superinfecting virus becoming the dominant viral population, as was also observed in gp41 Highlighter plot for this individual (Figure 1D).

Another example of almost complete dominance by the superinfecting virus is seen with ZM247F. This individual was initially infected by two variants that differ by 2.7% from the same donor, evidenced by two distinct branches of almost identical sequences [43] (Figure 3). Consistent with the Highlighter analysis (Figure 1E), all of the sequences amplified from the three-month time point cluster independently from the initial infecting viruses (red). These later sequences diverge over time and include recombinants with the initial infecting virus (see below), confirming the co-existence and genetic interaction of both the initial and superinfecting virus strains.

Figure 4 illustrates an example of what appears to be superinfection of the chronically infected male partner by his acutely infected spouse (ZM211M, Figure 4A), and superinfection of the newly infected female partner nine months later from an

outside source (ZM211F, Figure 4B). In this case the chronically infected partner, ZM211M (Figure 4A), has evidence of a distinct, diverse, cluster of *env* variants, at the time of his partner's seroconversion that represents the chronic viral population (blue). However, in contrast to the chronically infected partner in the other 2 cohabiting couples (ZM282 and ZM247), there is evidence for superinfection in the male at the time of his partner's seroconversion, with a subset of sequences that cluster closely with the woman's acute sequences (purple). Three months later, there is evidence of recombinant variants developing that contain a greater fraction of the man's sequence (see below). We interpret these findings to indicate that during acute infection, the woman partner transmitted her genetically distinct virus to her spouse, who died 6 months later. However, because plasma samples were not available prior to the woman's seroconversion time-point, it is possible that the male was infected by two phylogenetically distinct viruses and that one of these is the source of his spouse's (ZM211F) primary infection. Recombination analyses described below are most consistent, however, with superinfection of the male by his acutely infected partner.

ZM211F, the acutely infected partner, has a homogenous viral population at her earliest time point, which developed very limited diversity over the first 6 months (Figures 1F, and 4B). At the 9-month time point, ZM211F exhibits clear evidence of superinfection by a virus (red) that is genetically distinct from her partner's (black).

In order to rule out evidence that superinfections might have originated from contaminating sequences within the cohort, we analyzed the *env* SGA sequences from the three superinfection pairs in the context of contemporaneous sequences from the cohort (Figure 5A). In each case a distinct superinfecting genetic variant could be identified which segregated independently on the phylogenetic tree. The extra-cohort

origin of these superinfecting strains was further supported by a phylogenetic analysis of gp41 population sequences for the viruses from all 22 couples (Figure 5B).

Recombination analysis using Highlighter tool

One consequence of superinfection can be the generation of novel unique recombinant viruses, and evidence for HIV-1 superinfection can be further supported in the 3 couples analyzed here by evaluation of the sequences for recombination. For each superinfected individual, parental viruses were selected by generating a consensus of full-length *env* SGA sequences from the time of seroconversion and choosing a full-length *env* SGA amplicon sequence that matched the consensus sequence (filled blue circle). Similarly, parental superinfecting viruses were selected by comparing the chosen viral *env* sequence against all *env* sequences at the time of superinfection and selecting the superinfecting *env* sequence with the greatest pairwise distance from the seroconversion virus (filled red circle).

Figure 6A clearly shows that for each of the selected ZM282M sequences recombinant variants have been generated (purple stars, Figure 2). In the ZM282M_10 recombinant sequence for example, the C-terminal region of gp120 and N-terminus of gp41 of the initially infecting virus (blue) have been replaced by the superinfecting virus sequences (red). By contrast, as might be predicted from its position on the N-J tree, ZM282M_12 consists almost entirely of superinfecting virus sequences, with only small regions of gp120 and the C-terminus of gp41 originating in the initial acutely infecting virus.

In the case of ZM247F (Figure 6B), however, despite very distinct recombination patterns across the *env* gene in the three cases chosen, a recombination break point within the C-terminal domain of gp41 (residue 2200), first observed at 12 months (F_12), was conserved in the 15 and 21 month sequences even though these variants were located in distinct clusters on the N-J tree. This suggests that this recombination event may have conferred a specific fitness advantage.

In couple ZM211, we compared presumed recombinant viruses in the chronically infected partner ZM211M with virus sequences from both the ZM211M quasi-species (blue circle, Figure 4A) and the ZM211F initially infecting viruses (red circle, Figure 4A). In the latter case (Figure 6C) the recombinant viruses clustering closely to the newly infecting ZM211F founder sequence (purple stars, Figure 4A) exhibited discrete recombination events, but also showed evidence of conserved recombinant regions derived from ZM211M at the very N-terminus of gp120.

Finally, a comparison of three putative recombinants in the superinfecting population of ZM211F (purple stars in Figure 4B) to the initial virus and the superinfecting strain at 9 months (ZM211F_9) revealed clear evidence of recombination in the superinfecting strain with acquisition of *env* sequences from the initially infecting virus at the N-terminus of gp120 and the C-terminal domain of gp41 being evident at 12 and 21 months. Thus in each of the four superinfected individuals, we observed extensive recombination with evidence for co-existence of diverse recombinants at time points subsequent to superinfection.

Discussion

Understanding the mechanism for HIV-1 superinfection is crucial to the development of an HIV vaccine in order to prevent HIV-1 acquisition in a naïve host, since HIV-1 superinfection calls into question the idea that a robust primary immune response to HIV-1 infection provides some immunological protection from re-infection with a heterologous HIV-1. Superinfections occurring in heterosexual cohabiting pairs have not been studied in detail or longitudinally, despite the fact that most primary infections occur in HIV-1 discordant couples [40,41]. Though discordant couples are considered 'high risk,' they are not typically thought to be exposed to as many different viruses as sex workers or intravenous drug users [31,47]. For this reason, it might be predicted that superinfection would be observed at lower frequency and would occur predominantly between individuals within a couple; however, this was not the case. In the 22 newly infected partners, who had acquired HIV outside the marriage, we observed a frequency of superinfection in these individuals in the first year of follow up that was similar to that of primary infection (13.6% vs. 7.8%, $p > 0.05$). Even though we excluded couples in which the chronically infected partners had viral loads lower than 1000 from this study, all of the superinfections in the seroconverting partner originated from a non-spousal partner. Thus, it is clear that these couples are a higher risk subset of the cohort with exposure to HIV-1 infection outside the main partnership. The very limited frequency of intra-couple superinfection (1/4) studied here in part reflects continued safe sexual practices within the couple, since greater than 95% of reported sexual activity was with a condom. Nevertheless, significant numbers of non-condom exposures did occur (104/2146) in 19 of the 22 newly infected partners who did not become superinfected. Although self-report of external sexual activity is clearly underreported [37], it seems unlikely that for each of the three superinfected individuals, the number of

unprotected extra-marital exposures would exceed the number within the marriage. Moreover, with an adult seroprevalence rate of ~20% in Zambia, extramarital exposures should in a majority of instances be with seronegative individuals and therefore present less of a risk of potential superinfection than with the known seropositive partner. Interestingly, a similar lack of intra-couple superinfection has been observed in a recent study of 11 seroconcordant couples infected with disparate viruses in Uganda [48].

One factor that could influence susceptibility to superinfection is the presence of sexually transmitted diseases. Since genital infections and ulcers break down mucosal barriers and contribute to increased risk for primary HIV infection [42,49,50]. In the analysis of behavioral characteristics and clinical signs, the factors that trended toward significance were the presence of GUD on physical exam and RPR positivity in the superinfected group as compared to the non-superinfected group, although 7/19 non-superinfected individuals did have GUD. Previous studies in this Zambian cohort have shown a 2–3 fold increase in risk of HIV-1 infection in uninfected partners with GUD, after correction for viral load in their chronically infected partner [51-53]. In contrast to this higher-risk group, longitudinal *gag*, *pol*, and *nef* gene sequence data from 80 epidemiologically linked transmission pairs in the cohort (where transmission was from the cohabiting spousal partner) in the first two years of follow-up have not demonstrated any evidence of superinfection from non-spousal partners (data not shown), consistent with a lower frequency of extra-marital sexual activity in this cohort subset.

Despite the fact that a majority of the acutely infected individuals in this study of ZEHRP transmission pairs have >2 years of follow-up, HIV-1 superinfection was observed within the first year of follow-up in each of the 3 acutely superinfected individuals. This is consistent with recent studies of intra-subtype superinfection in subtype B infected individuals, where in one case mathematical modeling indicated a 21-

fold reduction of superinfection after 1 year of infection [25], and in a second case, a retrospective analysis of individuals in the San Diego and Los Angeles Acute HIV Infection and Early Disease Research Programs demonstrated 3 cases of superinfection within 13 months of seroconversion [17]. In contrast, the timing of superinfection in a subtype A commercial sex worker cohort appears less constrained, with superinfection detected as late as 5 years after primary infection [26].

The analysis of longitudinal *env* sequences, amplified by the SGA approach, for each of the individuals identified through degenerate base analysis allowed the definitive resolution of both the timing and nature of superinfection. In each of the three recent seroconversion cases a distinct superinfecting genetic variant could be identified which segregated independently on the phylogenetic tree (Figure 5A). Recombination between the primary infection variant and the superinfecting variant was observed in each case; and at some time points, consistent with the Highlighter analyses of population sequences, these recombinants became the dominant variant in the circulating virus population. Interestingly, we observed the conservation of recombination break-points within different variants in an individual over several months, suggesting that recombinant viruses with these particular sequence mixes possess fitness benefits over either the initial or the superinfecting strain. This is consistent with the observation of Streeck et al., [36], who showed that recombination between initial and superinfecting viruses could accelerate immunological escape from cellular immune responses. In a more global sense, the selection of mixed genotypes with enhanced population fitness is evidenced by the numerous circulating recombinant forms of HIV-1 resulting from dual infection of individuals [9,33,34], which clearly contribute to the overall diversity of a virus population. Additional studies will be required to fully characterize the basis of recombinant virus selection in the subtype C infected individuals under study here.

The SGA analysis of viral sequences bolstered our interpretation that ZM211M was superinfected from his spousal partner, ZM211F, during her acute seroconversion. At the time of her seroconversion, ZM211M has two dominant and distinct quasispecies with limited evidence for recombination between them. In contrast at month 3, a distinct population of recombinant viruses arises. This is consistent with superinfection of ZM211M during his spouse's acute viremia (viral load greater than 750,000), followed by the emergence of recombinants. Moreover, shortly after the probable superinfection, the viral load of ZM211M increased 10-fold and he is deceased within 6 months.

Determining why HIV-1 does or does not superinfect an exposed individual will be crucial to understanding the nature of an immune response that is capable of preventing *de novo* infection. Given the considerable antigenic dissimilarities between subtypes, we might not expect that initial infection by one subtype of HIV-1 would provide significant immune protection against other subtypes; on the other hand we might expect there to be some protection from reinfection of infected patients by more closely related HIV-1 strains of the same subtype. This does not appear to be the case during the first year of infection in the subtype C infected individuals studied here, where rates of intra-subtype superinfection in the first year of study were similar to those of primary infection [39]. However, it is of interest that in the three individuals that are superinfected, little variation in *env* sequences is observed in the period prior to the superinfection event, suggesting that there may be limited neutralizing antibody pressure on the founder virus. Indeed, preliminary studies indicate the absence of potent neutralizing antibody responses to the founder virus at the visit prior to superinfection (D. Basu et al., unpublished). It will be of interest to determine whether there is a more potent neutralizing antibody response in the non-superinfected individuals who also report extra-marital contact. Moreover, given that in this study each partner in the

couple is infected with a different strain of subtype C HIV-1, it is possible that repeated exposure to a partner's HIV-1 strain could stimulate the development of HIV-1 specific immune responses and that this might have provided protection against intra-couple superinfection. This type of immune stimulation with boosting of the cellular immune response has been reported to occur in subtype B infected men who have sex with men [23].

The existence of HIV-1 superinfection presents an obstacle to develop a vaccine to prevent primary infection with HIV-1. With technologies such as next-generation sequencing being employed to detect HIV-1 superinfection [48], the detection of very small viral sub-populations at a given time point will increase resolution. There are behavioral and clinical aspects (e.g. circumcision, genital ulcers) that influence this phenomenon but there are likely immunologic correlates that render some individuals more susceptible to superinfection. Continued study of HIV-1 superinfection within cohabiting heterosexual couples can provide insights into such correlates in the context of a potentially highly susceptible and relatively low-risk cohort type.

Acknowledgements

We would like to acknowledge the staff and volunteers of the Zambia Emory HIV Research Project. We would like to thank Dr. Jesse T. Jacob, Amanda Tichacek, and Naw Htee Khu for assistance with the behavioral statistics.

Financial support: The work was supported by grants from the National Institutes of Health (AI-51231; MH-66767; HD-40951) and the International AIDS Vaccine Initiative. The Virology Core of the Emory Center for AIDS Research (P30 AI050409) provided support for viral load testing. NIH AI-0678501 supported PTH. An NRSA Institutional Postdoctoral Training Grant T32 AI-007470 through the Emory Microbiology and Molecular Genetics Program and National Institutes of Health/National Center for Research Resources KL2 RR-025009 supported CSK. The Fogarty AITRP grant (D43 TW001042) sponsored WK. Funding institutions played no role in the conduct of these studies or in the preparation, review or approval of the manuscript.

Nucleotide sequences: GenBank SGA accession numbers for ZM247: EU166779-EU166856

GenBank population sequencing for gp41: GU827726-GU827976

Table 1. Reported sexual activity of newly infected partner in 12 months post-primary infection

Variable	Not Superinfected (n=19)	Superinfected (n=3)	p-value
Age	28	26	0.41
Female gender	47%	66%	0.43
Reported genital ulcer/infection	7/19	3/3	0.01
Sex with partner/no condom, mean (range)#	5.5 (0–26)	9.7 (2–24)	0.57
Sex with partner/with condom, mean (range)#	107.4 (10–322)	67 (11–112)	0.51
Sex/non-spousal partner (contacts; fraction reporting exposure)	5; (3/19)	11; (1/3)*	0.27
RPR positivity	2/19	2/3	0.02
Trichomonas (in female partner)	4/19	1/3	0.31
Pregnancy	1/9	0/2	0.70

* self report – rare for women
# mean value/yr in first year

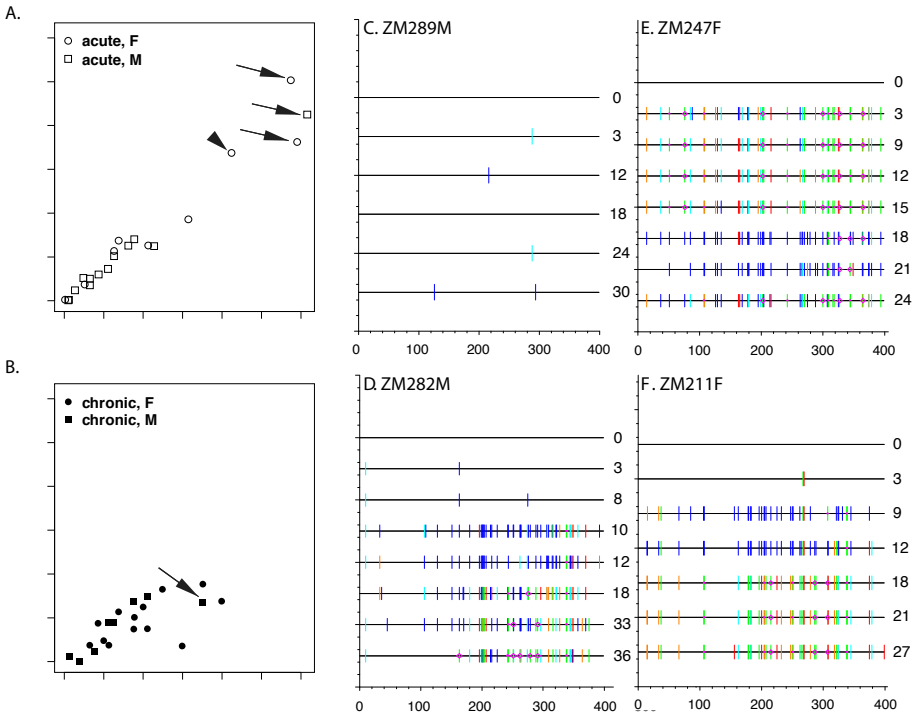

Figure 1. Sequence and Highlighter Analysis of Longitudinal Samples Provides Evidence for Superinfection. Comparison of maximum sequence divergence in gp41 versus the maximum number of degenerate bases at any time point within acutely infected individuals (**A**) and chronically infected individuals (**B**). The maximum percentage of degenerate bases is plotted on the y-axis; the maximum percentage of genetic distance is plotted along the x-axis. Black arrows indicate superinfected subjects; arrowhead indicates a subject co-infected with two variants from a single donor. Highlighter plots for gp41 sequences of ZM289M (**C**), ZM282M (**D**), ZM247F (**E**), and ZM211F (**F**) sampled at 0 to 36 months post-seroconversion (shown on right of plot). The sequence at each time point is compared to the initial infecting HIV-1 gp41 sequence. Tick marks denote nucleotide changes from the seroconversion sequence (T = red, A = green, C = blue, G = yellow), with dark blue indicating degenerate bases (See Methods).

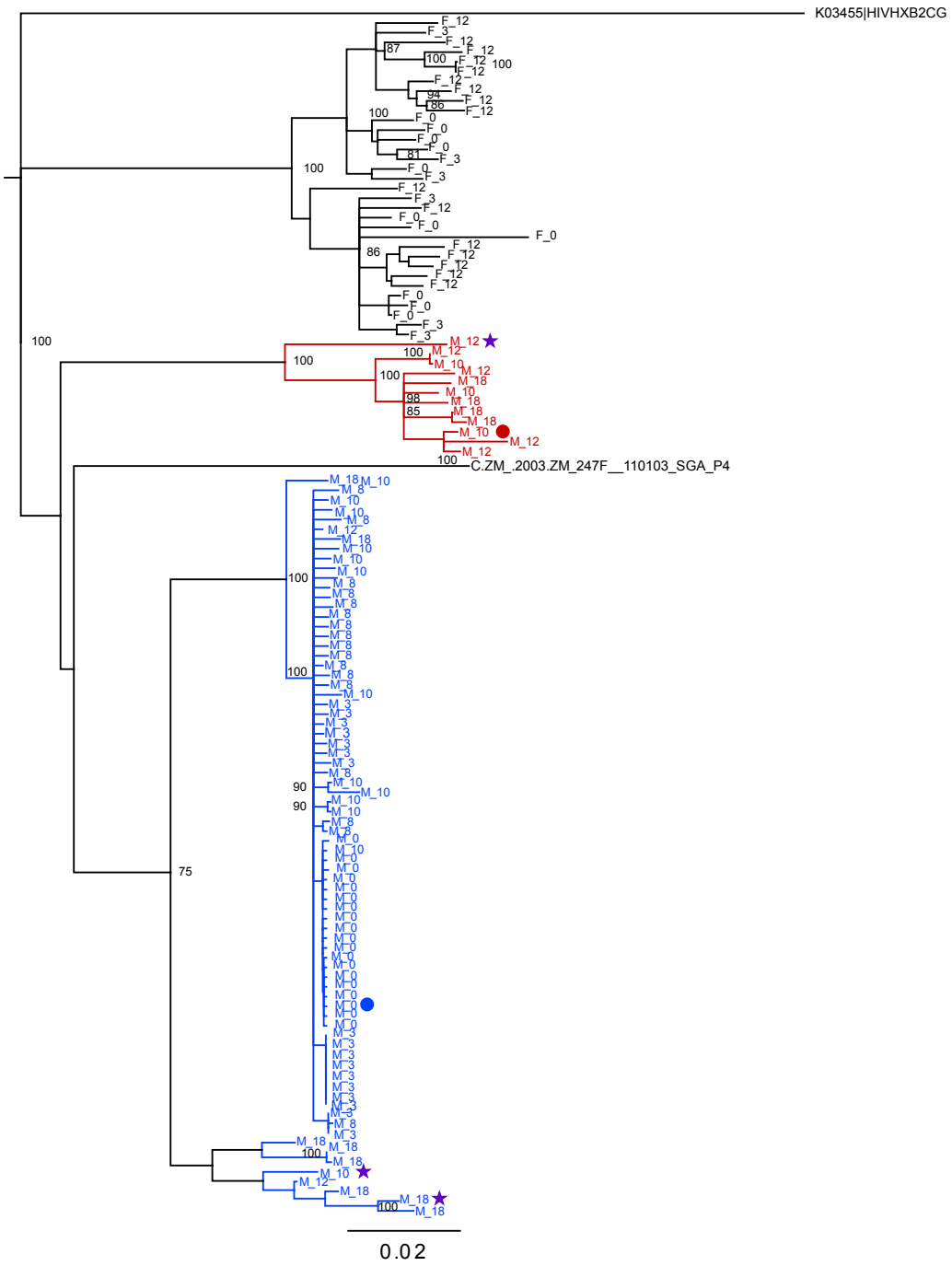

Figure 2. Neighbor-joining tree of full-length SGA env sequences for ZM282M. Blue and red sequences represent the initial infection and superinfection sequences, respectively, from the acutely infected subject ZM282M; black sequences are derived from the epidemiologically unlinked cohabiting partner ZM282F, who is chronically infected. The

time points are indicated along with M or F for each sequence (i.e. M8 is the acutely infected Z282M at 8 months post seroconversion). The ZM282F “0” time point corresponds to seroconversion of ZM282M. The duration of infection for ZM282F is not known. Bootstrap values >80 are considered statistically significant. Sequences denoted by circles indicate the parental sequences (blue, red circle) and those denoted by stars identify potential recombinant daughter sequences that were used for recombination analyses (below).

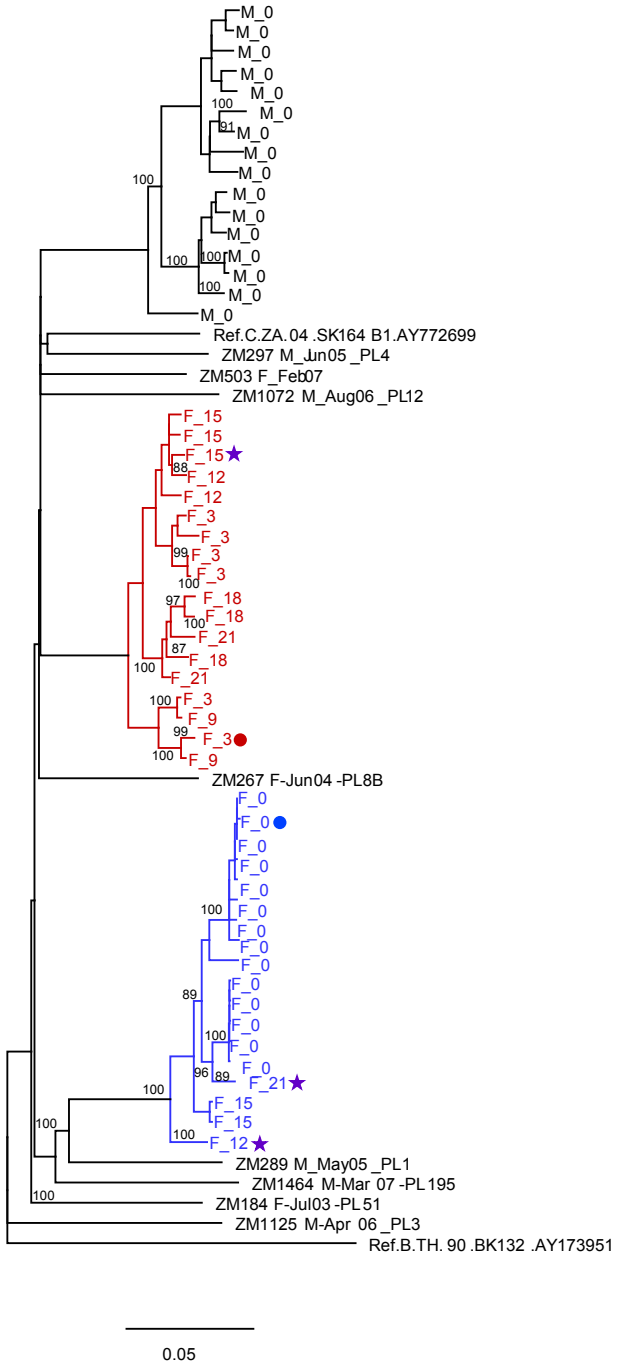

Figure 3. Neighbor-joining tree representing full-length SGA env sequences for ZM247F. Blue and red sequences represent the env SGA sequences from acutely infected ZM247F (blue) and superinfected (red) viral sequences. Black sequences are derived from ZM247M, the epidemiologically unlinked chronically infected partner. Bootstrap

values >80 are considered statistically significant. Sequences denoted by circles indicate the parental sequences (blue, red circle), and stars denote potential recombinant daughter sequences (purple stars) that were used for recombination analyses (below).

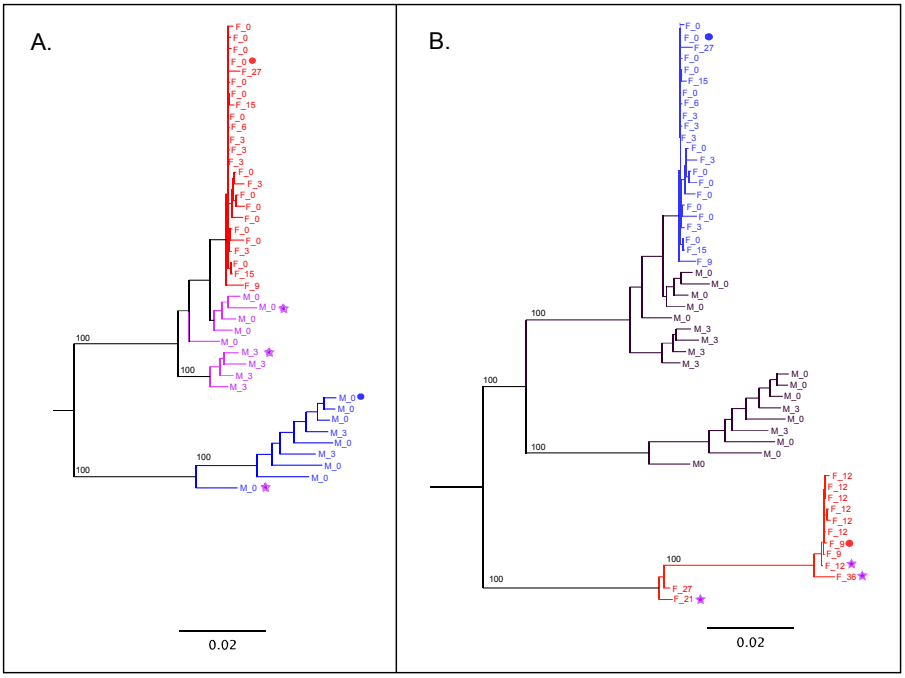

Figure 4. Neighbor-joining tree of full-length SGA env sequences for ZM211M and ZM211F. (**A**) The chronically infected ZM211M sequences are depicted in blue, and the superinfecting ZM211F sequences are depicted in red, while those in purple represent potential recombinant sequences between the blue and red sequences. (**B**) An expanded phylogenetic tree showing time points 0–36 months for the acutely infected ZM211F initial infecting virus (blue) is distinct from ZM211M (black). ZM211F was superinfected twice: first at 9 months (red), and then again at 30 months (green), both by non-spousal transmissions with two different individuals. Sequences denoted by circles indicate the parental sequences (blue, red circle) and stars denote potential recombinant daughter sequences (purple stars) that were used for recombination analyses described below.

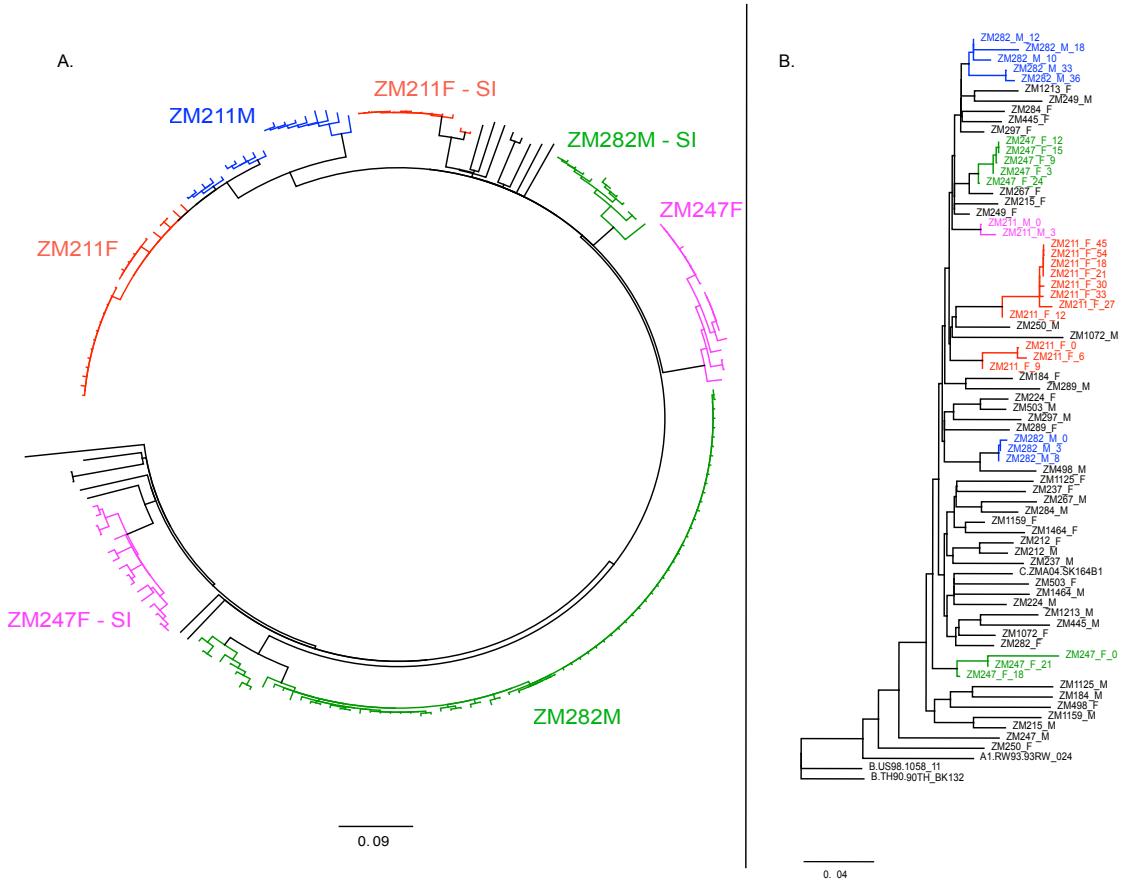

Figure 5. Neighbor-joining trees of SGA env and population gp41 sequences for the cohort. (**A**) Neighbor-joining phylogenetic tree of single genome amplified *env* gene sequences from each superinfected individual in the context of Zambian sub-type C *env* sequences. The Zambian subtype C sequences include twelve of the non-superinfected, newly infected individuals from this study. Superinfected individuals are assigned discrete colors and the superinfecting quasispecies is denoted by SI. (**B**) Neighbor-joining tree of gp41 sequences for all 22 couples. Superinfected individuals are assigned discrete colors.

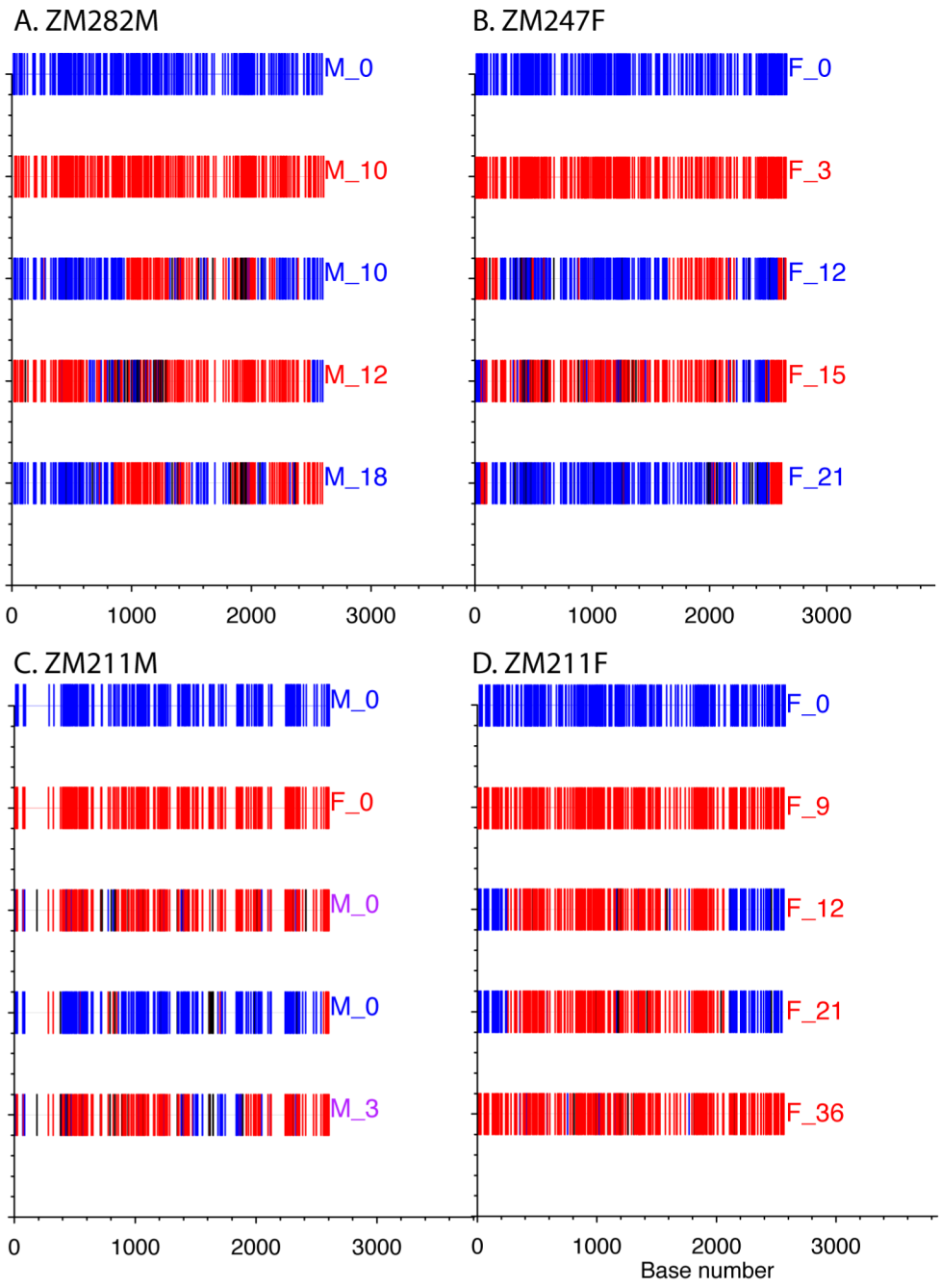

Figure 6. Highlighter recombination plots of full-length env for each individual who was superinfected. Highlighter recombination analysis of sequences from ZM282M (**A**), ZM247F (**B**), and ZM211M (**C**) and ZM211F (**D**). The parental virus sequence from the acutely infected or a representative parental sequence from the chronically infected partner (ZM211M) are shown in blue in the first bar of each panel. The superinfecting

parental sequence shown in red is the second bar. The presumptive recombinant daughter sequences from three representative time points are shown below the parents for each case of superinfection.

Table S1. Demographic, clinical and behavioral data on 22 Zambian unlinked transmission pairs

Couple ID	Sex	Age[1]	Acute VL[2]	Index VL	Sub-type	GUD[3]	STI[3]	Trich	RPR	Circum-cision	Preg	Sex with condom[4]	Sex w/o condom[4]	Other partner[5]
ZM184	F	30	71,290	147,239	C	Yes	Yes	Yes	No	No	No	39	9	Yes
ZM211	**F**	**30**	**2,640**	**96,689**	**C**	**No**	**Yes**	**No**	**No**	**No**	**No**	**11**	**3**	**No**
ZM212	F	37	10,548	750,000	C	No	No	No	No	No	No	322	5	No
ZM215	F	24	241,190	ND	C	No	No	No	No	No	No	155	26	No
ZM224	F	22	624,803	17,252	C	Yes	Yes	No	No	No	No	24	0	No
ZM237	M	43	44,870	1,323	C	Yes	Yes	No	No	No	NA	41	4	No
ZM247	**F**	**23**	**750,000**	**53,854**	**C**	**No**	**Yes**	**Yes**	**Yes**	**No**	**No**	**112**	**26**	**No**
ZM249	M	30	750,000	ND	C	Yes	No	No	No	No	NA	145	25	Yes
ZM250	M	22	65,260	46,215	C	No	No	No	Yes	No	NA	133	0	Yes
ZM267	F	35	750,000	180,355	C	No	Yes	No	No	No	Yes	188	8	No
ZM282	**M**	**25**	**750,000**	**56,700**	**C**	**Yes**	**Yes**	**No**	**Yes**	**No**	**NA**	**78**	**2**	**Yes**
ZM284	M	36	750,000	91,583	C	No	No	No	No	No	NA	97	1	No
ZM289	M	27	399,737	6,692	C	No	No	Yes	No	No	NA	75	8	No
ZM297	M	50	8,687	ND	C	No	No	No	No	No	NA	108	3	No
ZM445	M	32	11,660	22,710	C	No	No	No	No	No	NA	69	4	No
ZM498	F	41	14,220	ND	C	No	Yes	Yes	No	No	No	52	0	No
ZM503	F	22	25,800	ND	C	No	No	No	No	No	No	65	0	No
ZM1072	M	28	36,200	400	C	No	No	No	No	No	NA	144	0	No
ZM1125	M	31	2,006	2,280	C	No	No	No	No	No	NA	153	10	No
ZM1159	M	29	110,220	493	C	No	No	Yes	No	No	NA	90	1	No
ZM1213	M	30	76,286	68,535	C	Yes	No	No	Yes	No	NA	74	5	No
ZM1464	M	29	44,500	34,100	C	No	No	No	No	No	NA	68	0	No

Bolded individuals were confirmed to be superinfected

[1]Age at enrollment

[2]Viral Loads were collected for first antibody positive dates

[3]Genital ulcer disease or STI in 12 months

[4]Sexual data is cumulative events for 12 months

[5]Other partner in the last 12 months

ND=not done, NA – Not applicable

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Chapter 3

HIV-1 subtype C superinfected individuals mount low autologous neutralizing antibody responses prior to intrasubtype superinfection

Published in *Retrovirology, 2012, 9:76*.

Debby Basu performed all of the experiments in this manuscript (Figures 2-8, Supplementary Figures 1-4), except as follows:

Colleen S. Kraft generated the *env* single genome amplicons from superinfected individuals (Figure 1)

Peter T. Hraber performed the analysis of *env* diversification (Figure 1)

Debby Basu and Eric Hunter wrote the manuscript.

Abstract

Background

The potential role of antibodies in protection against intra-subtype HIV-1 superinfection remains to be understood. We compared the early neutralizing antibody (NAb) responses in three individuals, who were superinfected within one year of primary infection, to ten matched non-superinfected controls from a Zambian cohort of subtype C transmission cases. Sequence analysis of single genome amplified full-length *envs* from a previous study showed limited diversification in the individuals who became superinfected with the same HIV-1 subtype within year one post-seroconversion. We hypothesized that this reflected a blunted NAb response, which may have made these individuals more susceptible to superinfection.

Results

Neutralization assays showed that autologous plasma NAb responses to the earliest, and in some cases transmitted/founder, virus were delayed and had low to undetectable titers in all three superinfected individuals prior to superinfection. In contrast, NAb with a median IC50 titer of 1896 were detected as early as three months post-seroconversion in non-superinfected controls. Early plasma NAbs in all subjects showed limited but variable levels of heterologous neutralization breadth. Superinfected individuals also exhibited a trend toward lower levels of gp120- and V1V2-specific IgG binding antibodies but higher gp120-specific plasma IgA binding antibodies.

Conclusions

These data suggest that the lack of development of IgG antibodies, as reflected in autologous NAbs as well as gp120 and V1V2 binding antibodies to the primary infection

virus, combined with potentially competing, non-protective IgA antibodies, may increase susceptibility to superinfection in the context of settings where a single HIV-1 subtype predominates.

Keywords: HIV-1 superinfection, subtype C, neutralizing antibodies, HIV-1 transmission, HIV-1 dual infection

Background

To develop a cross-protective HIV-1 vaccine that provides immunological breadth against multiple strains, a comprehensive understanding of the immunologic and virologic interactions that occur during HIV-1 superinfection in clinically relevant populations is critical. HIV-1 superinfection refers to re-infection with a heterologous HIV-1 variant in an HIV-infected individual, who has had the opportunity to mount an immune response to the primary infection [1]. Elucidating immunological factors that may prevent superinfection (despite exposure to virus) will inform our understanding of possible correlates of protection from *de novo* infection, as well as what factors may contribute towards a successful vaccine-induced immune response.

Non-human primate studies have shown that neutralizing antibodies (NAbs) and passive transfer of broadly cross-reactive monoclonal antibodies can confer protection against simian-human immunodeficiency virus (SHIV) infection [2-7]. Results of the RV144 vaccine trial have also supported that specific humoral responses, including higher levels of V1V2-binding IgG antibodies, may have contributed to protection from primary HIV-1 infection in uninfected vaccinees, and that higher anti-Env plasma IgA levels may have contributed towards risk of primary HIV-1 infection in vaccinees [8,9]. Another approach to address the potential contribution of antibodies to protection from primary HIV-1 infection is to evaluate whether they decrease susceptibility to superinfection. Specifically, antibody responses in individuals who become superinfected versus those who are similarly exposed to exogenous virus but remain singly-infected can be evaluated for differences that may confer protection. NAb responses in the context of superinfection have been studied in subtype A [10,11] and B [12,13] HIV-1 infection, in addition to settings where multiple clades and recombinant species are common [10,14]. However, to date, there is no clear resolution of whether NAbs could

play a role in modulating susceptibility to superinfection or whether trends observed in such studies were context-dependent.

Studies of a commercial sex worker (CSW) cohort in Mombasa, Kenya have shown HIV-1 intra- and inter-clade superinfections to occur during both early and chronic infection [10,11,15], with no significant difference in heterologous neutralization breadth or potency against a wide panel of cross-clade pseudoviruses in superinfected individuals versus non-superinfected matched controls prior to superinfection [10]. In contrast, intrasubtype B superinfections in an MSM cohort in San Diego have been shown to occur primarily during the first year of infection, with lower baseline NAb breadth to two lab-adapted strains and autologous viruses isolated from pre-superinfection plasma [13]. Other subtype B studies have also shown, through mathematical modeling, a 21-fold reduction in the rate of superinfection after the first year of infection [16], consistent with some change in susceptibility. However, despite the fact that most new seroconversions in adults occur in heterosexual discordant couples [17] in subtype C endemic areas, the dynamics of early humoral responses in the context of superinfection in this cohort type have not been thoroughly examined.

We previously reported, from an HIV-1 discordant couple cohort in Lusaka, Zambia [17,18], a longitudinal study of 22 newly infected individuals, where three superinfection cases were identified (13.6%). HIV-1 superinfection was initially identified using a combination of screening methods with final confirmation by sequencing of single-genome amplified *env* genes [19]. In each case, superinfection was by a virus from a non-spousal partner during the first year of infection. In all cases, the superinfecting variant predominated and extensive recombination between superinfecting and initial variants occurred after the superinfection event. The finding that superinfections were commonly seen during early infection from outside partners implicated potential roles

for sexual risk behavior [19] and early immune responses in modulating superinfection outcomes in this cohort. We have therefore investigated early antibody responses in these three intrasubtype C superinfected individuals and 10 of the 19 non-superinfected individuals from the same Zambian cohort of heterosexual couples.

These studies show that autologous plasma NAb titers to the early/founder viruses were low to undetectable in all three superinfected individuals prior to superinfection, whereas the majority of non-superinfected controls mounted early and strong autologous responses to the early/founder Env as early as three months post-seroconversion. Similarly, gp120 and V1V2-specific IgG antibody titers were higher in matched controls while gp120-specific plasma IgA titers were higher in two of three superinfected individuals, suggesting that reduced IgG and increased IgA humoral immune responses may influence the risk of superinfection in this subtype C cohort.

Materials and Methods

Study subjects

Heterosexual cohabiting couples in serodiscordant relationships were followed by the Zambia-Emory HIV Research Project (ZEHRP) in Lusaka, Zambia. ZEHRP provides couples' voluntary counseling and testing as well as condom provision, general health care, and family planning counseling to participating couples [18,39]. These strategies have been shown to effectively reduce transmission rates between partners in participating countries [17]. Couples are tested for HIV-positivity, as previously published [18,39-41]. Seroconversion of the initially uninfected partner occurs approximately 7-8% per year, and the new seroconverter is subsequently followed quarterly, with annual follow-up for the chronic partner. Plasma is collected at each visit from study participants, with informed consent and under human subject protocols approved by both the University of Zambia Research Ethics Committee and the Emory University Institutional Review Board.

Epidemiological linkage was determined as previously published; unlinked transmission pairs, in which the negative partner in the serodiscordant couple became infected from an individual outside of the partnership, were identified [42]. Twenty-two unlinked couples were chosen and screened for superinfection for up to 5.5 years of follow-up [19]. Viral RNA was extracted from plasma samples at the time of seroconversion and longitudinally thereafter using the QiaAMP Viral Mini Extraction kit for phylogenetic evaluation of viral sequences. Identified superinfected individuals were matched to 10 selected non-superinfected controls from the study [19] based on 1) time from the last seronegative to the first antigen or antibody positive sample, 2) seroconversion viral load, 3) subtype of infection and 4) occurrence of seroconversions within the same five-

year interval. When possible, superinfected controls were matched to non-superinfected controls that had self-reported extra-marital (outside) partnerships (ZM249M, ZM184F). Underreporting of sexual exposures is common in this Zambian cohort [43], and in this study, all initial infections were identified as being epidemiologically unlinked, implicating risk for outside partnerships despite lack of self-reported cases.

Superinfection detection and characterization

Superinfections were identified by a combination of screening methods including phylogenetic analysis of gp41 and p24 gag population sequences, heteroduplex mobility assays using gp41 amplicons, and degenerate base counting of population sequences [19]. If individuals showed preliminary evidence of superinfection, longitudinal full-length *env* single genome amplification was performed using nested PCR [19,29,44,45] in order to confirm the presence of superinfection by phylogenetic analysis [19]. Sanger DNA sequencing was performed by the University of Alabama at Birmingham Center for AIDS Research (P30 A127767) DNA Sequencing Shared Facility using a 3730xl DNA Analyzer and BigDye Terminator v3.1 chemistry.

Phylogenetic analysis

Sequences were assembled and evaluated using Sequencher 4.10 (Gene Codes Corporation, Ann Arbor, MI) and Geneious Pro 5.4.6 (Biomatters Ltd, Auckland, New Zealand). Sequence alignments and neighbor-joining phylogenetic trees were generated using the Tamura-Nei genetic distance model with the bootstrap resampling method. Superinfecting variant gp41 sequences consistently had greater than 11% pairwise distance from the initial founder virus [19]. Single genome sequences of full-length *envs*

were amplified from plasma samples from all superinfected individuals from the time of seroconversion and longitudinally for the first year. All *envs* were processed as described above for phylogenetic analysis. Highlighter plots (LANL HIV Sequence Database) were used to evaluate longitudinal evolution of full-length *env* sequences from the determined early/founder sequence (see below). These plots were generated using aligned nucleotide sequences of the initial infection sequences only; each colored hatch-mark represents a single nucleotide change from the early/founder *env* sequence (red = T, green = A, orange = G, light blue = C). For longitudinal pairwise distance analyses, we used codon-aligned sequences to generate seroconversion consensus sequences, then computed raw pairwise distances to this consensus for each sequence sampled using R (version 2.15.0) with the package 'ape' (version 3.0-3) [46,47].

Amplification and cloning of full-length env genes

Phusion HotStart II Hi-Fidelity DNA polymerase (Finnzymes, Thermo Scientific) was used to amplify an average of 10 single genome full-length *env* amplicons per subject from plasma by nested PCR, as described elsewhere [45]. These amplicons were obtained from the time of seroconversion (Table 1, Additional file 1: Figure S1), and a sequence alignment was generated to establish the consensus from this time point. The amplicons whose sequences matched this consensus were typically representative of the founder virus envelope glycoproteins (Table 1, Additional file 1: Figure S1). Superinfecting virus amplicons were selected by comparing the chosen early/founder virus sequence against all *env* sequences at the time of superinfection detection and selecting the superinfecting *env* variant with the greatest pairwise distance from the early/founder virus *env* sequence [19]. These *env* genes were then directionally cloned using the pcDNA 3.1 Directional TOPO/v-His expression plasmid (Invitrogen), as previously described

[21,22,29]. All clones were tested for biological function, sequenced, and co-transfected with *env*-defective subtype B provirus SG3ΔEnv into 293 T cells using FuGENE HD (Roche) to generate envelope pseudoviruses carrying patient-derived *env* genes [21-24,29]. Envelope pseudoviruses were harvested 48-hours post-transfection. JC53BL-13 (TZM-bl) cells were infected at five-fold serial dilutions of virus for 48 hours for viral titering, and infectious units were determined through *β*-galactosidase staining and counting positive infected blue foci, as previously described [21-24,29].

Neutralization assays & calculation of IC50 titers

Neutralization assays using the indicator cell line JC53BL-13 (TZM-bl) were performed, as previously described [21-25,29]. Briefly, two thousand infectious units of envelope pseudoviruses in 3.5% FBS DMEM with 40 μg/ml DEAE-Dextran were incubated with five-fold serial dilutions of heat-inactivated patient plasma. Normal human plasma (NHP) was used to maintain an overall 10% concentration of plasma [22,25,29]. The virus-plasma mixture was added to seeded JC53BL-13 cells (plated and cultured overnight at 37°C) and incubated at 37°C for 48 hours, as previously described [22-25,29]. Cells were then lysed and luciferase was read for each well; luminescence was recorded accounting for background. Percent viral infectivity and correlating neutralization IC50 values (representing plasma dilution resulting in 50% viral infectivity) were determined using a linear-regression-least squares fit method, as described elsewhere [21-24,29]. For example, if 50% viral infectivity was achieved with a 1:2000 plasma dilution, the reported IC50 would be 2000. Each plasma-Env combination was tested in duplicate in each experiment and IC50 values shown represent mean IC50 values from at least two independent experiments.

For studies of autologous plasma neutralization, plasma dilutions started at 1:100, while for heterologous breadth studies, we started at a 1:20 plasma dilution in order to increase sensitivity for low titers of cross-reactive neutralizing antibodies. For the autologous neutralization studies, we tested the first post-seroconversion plasma and all subsequent plasma samples available within approximately the first year of infection. Seroconversion plasma was not tested for neutralization, and was assumed to be at our limit of detection for these studies at an IC50 of 100.

Heterologous neutralizing breadth and potency scores

The Subtype C HIV-1 Reference Panel of Env Clones [27,28] was obtained from NIH AIDS Reference and Reagent Program, and pre-superinfection plasma in superinfected individuals (and contemporaneous samples from non-superinfected controls) was evaluated for heterologous breadth to the 12-pseudovirus panel. After generating viral infectivity curves, neutralization IC50 values were calculated for each plasma-virus combination. Each combination was tested in duplicate, and IC50 values were averaged between the wells. Any IC50 values that were not reached at the lowest plasma dilution tested (1:20) were assigned an IC50 value of 10. Neutralization breadth scores were determined by adding the number of pseudoviruses in the panel neutralized at an IC50 greater than 20, while potency scores were determined by dividing the plasma-virus IC50 by the median IC50 per virus (against all plasma samples) and adding the scores for each plasma sample [10,48]. All potency score values were rounded to the nearest integer. In one case (ZM249M), because plasma was tested against an autologous envelope clone in the panel, IC50 values from this plasma-Env combination were discarded from the calculations for breadth and potency scores.

gp120 binding ELISA

gp120 binding ELISAs were performed in triplicate as previously described [49]. Briefly, 96-well ELISA plates were coated overnight with 100 μl (2 μg/ml) purified gp120 protein (GeneART) from the Zambian subtype C seroconverter ZM205F [23,29] at 4°C. Plates were then washed six times with PBS-T (PBS containing 0.1% Tween-20) and blocked with 200 μl B3T (150 mM NaCl, 50 mM Tris–HCl, 1 mM EDTA, 3.3% FBS, 2% BSA, 0.07% Tween-20 in 500 ml dd$H_2$0) for 1 hour at 37°C in a CO_2-free incubator. Plates were washed again, and 100 μl/well of five-fold serially diluted heat-inactivated plasma was incubated for 1 hour at 37°C. After washing six times with PBS-T, 100 μl of diluted secondary antibody (HRP goat anti-human IgG) was added to each well for 1 hour at 37°C. After a final wash six times with PBS-T, 100 μl of SureBlue TMB substrate solution (equilibrated to room temperature) was added to each well. Plates were incubated for 10 minutes at room temperature. In order to stop the reaction, 100 μl of 1 N H_2SO_4 was added/well, and plates were read at 450 nm absorbance with a Biotek Synergy plate reader and luminometer. Wells coated with gp120 protein alone were used as blank control wells and were subtracted from absorbance readings, as described below in the Statistical analysis methods section.

This protocol was also adapted to measure plasma IgA levels, with the following changes: Test plasma was depleted of IgG using the GE Healthcare Protein G HP/Ab Spin Trap and was subsequently serially diluted five-fold at a starting concentration of 1:25 in B3T blocking buffer. Results shown at 1:125 plasma dilution are representative of the trends observed across the serial dilution. The secondary antibody was changed to an HRP-conjugated goat anti-human IgA antibody (InvivoGen). This assay was performed in duplicate with normal human plasma (NHP), autologous (ZM205F) 31-month plasma and Subtype C pooled plasma controls. Wells coated with gp120 protein alone were

similarly used as blank control wells and were subtracted from absorbance readings.

V1V2 binding ELISA

A standard ELISA protocol was used to evaluate the presence of V1V2-specific IgG binding antibodies in heat-inactivated patient plasma (diluted 1:500 in 2% BLOTTO). Plates were coated with MuLVgp70-caseA2clB V1V2 [30] or MuLVgp70-conC V1V2 (consensus clade C) scaffolded proteins with MuLVgp70 carrier alone as a control. Positive absorbance was defined as absorbance greater than five times that of the uninfected normal human plasma control.

Statistical analysis

All statistical analyses compared responses between superinfected and non-superinfected groups. We performed the Wilcoxon rank sum test using the autologous neutralizing antibody IC50 titers obtained in the 5–8 month post-seroconversion time frame, which reflects the neutralizing antibody titers before superinfection. As subject ZM247F was superinfected at this time, for this subject alone we used the values obtained at 3 months post-seroconversion (which were equal to those obtained from 9- and 12-month plasma).

To evaluate differences in gp120-specific IgG binding antibody levels in pre-superinfection plasma in superinfected individuals and similar time points for controls, we evaluated gp120 binding ELISA data performed in triplicate. For each ELISA trial, we first found the baseline binding to purified gp120 protein – the lowest absorption value from the blank control wells. After adjusting for this baseline value, the experimental values were plotted, and the curve interpolated to find the titration corresponding to

50% of the highest binding absorption value of the curve. After all binding50 values were determined, we log-transformed the values for further analysis. A linear mixed effects model was used to determine whether the binding50 values were associated with superinfection status. Log-transformed binding50 values were used as the response variable, and the superinfection status was used as a predictor with fixed effect. The individual effects were modeled as random effects. We also calculated mean binding50 values for each test plasma and compared medians between superinfected and non-superinfected groups, using a Mann–Whitney test run in GraphPad Prism 5.0d. We similarly adjusted test plasma absorption values for background binding (as measured in the blank control wells) in the gp120-specific IgA ELISA, and compared median absorption values between groups amongst both trials using a Mann–Whitney test run in GraphPad Prism 5.0d.

Results

Limited Envelope (Env) diversification in the initially infecting virus prior to superinfection

In a previous study of superinfection within a subset of 22 newly infected individuals from the Zambia-Emory HIV Research Project (ZEHRP) discordant couple cohort [19], we identified three individuals who were superinfected from non-spousal partners within the first year of infection (detected 3–10 months post-seroconversion) with subtype C superinfecting variants.

As part of the previous study, we performed single genome amplification and sequencing of the *env* gene of the initially infecting virus at time points prior to superinfection. A phylogenetic evaluation of these longitudinal full-length *env* sequences showed remarkable homogeneity prior to superinfection for the two individuals in which superinfection was detected 9 and 10 months after primary infection. An example of this phenomenon is diagrammed via Highlighter plot for ZM282M in Figure 1A where few mutations were fixed over the first 10 months of infection. This is particularly clear when the pairwise distance of each amplicon sequence from that of the initial consensus sequence is plotted over time (Figure 1B-D). We observed a mean pairwise distance of only 0.1% prior to superinfection in both ZM282M and ZM211F, and less than 0.3% mean pairwise distance among all initially infecting variants of the three superinfected cases with respect to each individual's initial consensus sequence. This limited diversity contrasts with previous reports of approximately 1%/year during early infection [20]. Thus, from these panels, it is evident when superinfection was detectable (x-axis asterisk, Figure 1B-D) and that there was limited *env* sequence evolution prior to this event.

Neutralization of early/founder viruses during early infection

To evaluate early NAb responses in the three superinfected individuals, we matched the superinfected individuals to 10 of the 19 newly-infected non-superinfected unlinked partners. Matching parameters included subtype of infection, viral load at time of seroconversion, estimated time to infection, sample dates available, seroconversion in the same 5-year time span, and when possible, individuals self-reporting outside partnerships were included (Table 1). To test the hypothesis that lower titers of autologous NAb to the initial infection (early/founder Env) exist in the superinfected individuals, we utilized single genome amplification to obtain an average of 10 *env* amplicons (range 8–12) per individual (Additional file 1: Figure S1). After cloning of amplicons representing the consensus early/founder sequence (Table 1), we generated pseudoviruses carrying these Env glycoproteins for the three superinfected and the 10 singly-infected individuals. Using the standard JC53BL-13 (TZM-bl) neutralization assay [21-25], we tested autologous neutralization of the early/founder Envs by longitudinal plasma samples from the first year of infection. Viral infectivity curves plotting autologous neutralization of these early/founder variants were generated for each plasma time-point for each individual. Examples of these for the superinfected individuals and one non-superinfected control are shown in Figure 2A-D. Using these curves, plasma neutralizing antibody IC50 titers, which represent the plasma dilutions at which 50% of viral infectivities is achieved, were determined [22] over the course of the first year of infection for superinfected (Figure 3, dashed lines) and non-superinfected (non-SI) matched controls (Figure 3, solid lines).

Intrasubtype C superinfected individuals showed delayed kinetics and low-titer autologous NAb responses to the early/founder Env prior to detection of superinfection as compared to a majority of the non-superinfected controls, which had a median IC50

of 1896 as early as three-months post-seroconversion (Figure 3). Neutralization IC50 titers in the superinfected group were significantly lower at the pre-superinfection window of 5–8 months post-seroconversion compared to non-superinfected controls ($p = 0.039$). Although variable, neutralization kinetics and potency in the controls are similar to what has previously been shown [22,26], and are, therefore, an appropriate representation of typical early neutralization trends of subtype C infected seroconverters. A summary table (Figure 4A) of these autologous NAb IC50 titers highlights the early and strong responses seen in most non-superinfected controls, and the slower, low responses in superinfected individuals prior to superinfection. Non-superinfected controls that self-reported outside partnerships are bolded (Table 1 and Figure 4A).

Interestingly, IC50 titers in ZM211F and ZM282M, in which superinfection was detected at 9 and 10-months post-seroconversion, were very low (150 and <100, respectively) until the time point at which superinfection was detected (dashed line, Figure 2; asterisk, Figure 3), suggesting that infection with a distinct secondary variant may have elicited an immunological boost. In ZM247F, in which we detected superinfection at 3-months and an early predominance of the superinfecting variant [19], we could not detect titers of neutralizing antibodies greater than 100 to a founder variant even at 12 months (Figure 3); it was not until 15-months post-seroconversion, when evidence for re-emergence of the founder virus was observed [19], that neutralizing antibodies to the founder became measurable (IC50 of 1092; Additional file 1: Figure S2A).

Cross-neutralization of superinfecting viruses during early infection

To investigate further possible reasons for susceptibility to superinfection, we determined whether pre-superinfection plasma was capable of cross-neutralizing

pseudoviruses carrying Env glycoproteins isolated from the time at which superinfection was first detected. No evidence for cross-neutralization of the superinfecting variants by autologous pre-superinfection plasma existed for any of the three cases (Figure 4B). However, these superinfecting variants were neutralized by pooled subtype C plasma with IC50s of 210–572, suggesting they are not inherently neutralization resistant (Additional file 1: Figure S3).

In the case of early superinfection in ZM247F, although neutralization of the founder Env was not observed until after the first year of infection (Additional file 1: Figure S2A), we did observe preferential neutralization of the superinfecting variant with titers increasing from 6–12 months after superinfection was detected (Additional file 1: Figure S2B). This likely reflects the predominance of the superinfecting virus from 3–12 months post-seroconversion [19].

Heterologous neutralization breadth potential prior to superinfection

To evaluate whether superinfected individuals also lacked cross-neutralizing antibody breadth, we determined the ability of pre-superinfection plasma (as compared to contemporaneous plasma from controls) from early infection to neutralize a subtype C reference panel of 12 pseudoviruses. This panel included envelopes with tier 1 ("easiest" to neutralize) and tier 2 (more difficult to neutralize) sensitivities [27,28].

For these studies, the lowest plasma dilution was decreased to 1:20 to increase sensitivity of the assay. Pre-superinfection 6-month plasma from ZM211F was not able to neutralize to 50% any of the subtype C pseudoviruses tested (Additional file 1: Figure S4B, Figure 5). ZM247F 3-month plasma was capable of cross-neutralizing two pseudoviruses at very low IC50s (20 and 35) and ZM282M 8-month plasma cross-

neutralized seven pseudoviruses at IC50s greater than 20 but less than 100 (Additional file 1: Figure S4C and 4A, Figure 5). Non-superinfected controls showed similarly limited capacity for cross-neutralization (Figure 5). Interestingly, three non-superinfected controls (ZM284M, ZM503F, ZM1072M) that had the lowest autologous titers over the first year (Figure 4A), had some of the widest cross-neutralizing capabilities (Figure 5), indicating that heterologous breadth and autologous neutralization are not always correlated, consistent with previously published results from this cohort [22].

Analysis of gp120 and V1V2-loop binding antibody levels prior to superinfection

Recent analyses of the RV144 vaccine trial in Thailand have implicated non-neutralizing antibodies in protection from acquisition of HIV-1. We therefore measured levels of gp120-specific binding IgG antibodies in pre-superinfection plasma for superinfected individuals (Figure 6A; ZM282M: red, ZM211F: orange, ZM247F: green) and similar time points for non-superinfected controls (grey). Log_{10} values for 50% of maximum gp120 binding in this assay were determined and compared between superinfected and non-superinfected groups using a mixed linear effects model. While the plasma from superinfected individuals trended to lower titers, this was not significant ($p = 0.115$). Median values for 50% gp120-binding between the groups was also compared by Mann–Whitney test and showed similar results (Figure 6B, $p = 0.161$).

We also evaluated differences in gp120-specific plasma IgA levels in pre-superinfection plasma in superinfected individuals versus similar time points for matched controls, since high plasma IgA levels were correlated with risk of HIV-1 infection in the RV144 trial [8]. Strikingly, two of the three superinfected individuals had the highest levels of plasma IgA amongst all study participants (Figure 7). Only two of the ten non-superinfected controls elicited these gp120-specific plasma IgA responses

during early infection. These two matched controls were also the only non-superinfected individuals that had self-reported outside partnerships (a risk indicator of sexual exposure). When we compared the median absorption values between groups based on sexual exposure (superinfected individuals and non-superinfected individuals with self-reported outside partnerships against the non-superinfected individuals without self-reported outside partnerships) using a Mann–Whitney test we found that there was a statistically significant difference in plasma IgA levels between the groups ($p = 0.005$).

IgG antibodies binding to the V1V2-loop of gp120, which were correlated with protection in the RV144 vaccine trial [8,9], were also quantitated using the same MuLV gp70-V1V2 construct used in that study [30] as shown in Figure 8A. Plasma reactivity to a MuLVgp70-consensus clade C V1V2 construct was also tested (Figure 8B). None of the superinfected individuals showed evidence of binding antibodies to either V1V2 construct prior to or at the time of superinfection. In contrast, three of the ten non-superinfected controls showed evidence of antibodies capable of binding both constructs within the first 6 months of infection, with seven of the ten non-superinfected plasma samples binding to at least one V1V2 protein.

Discussion

In this study, we have shown that three intrasubtype C superinfected individuals, in whom superinfection was detected within the first year of infection, have low to undetectable titers of autologous NAbs to their early/founder Env prior to superinfection and as late as 8-months post-seroconversion. This is in sharp contrast to ten matched non-superinfected controls similarly evaluated for neutralization of early/founder variants over the first year of infection, of which a majority mounted very potent neutralizing activities. This occurred as early as three-months post-seroconversion, when the median IC50 was 1896. Despite the small size of this study, the differences in autologous NAb titers were significantly different between the two groups ($p = 0.039$), and suggest that slower development of a humoral immune response increased susceptibility to intra-subtype superinfection in this cohort.

This result is consistent with a previous study of a subtype B MSM cohort, where low titers of autologous and heterologous NAbs were observed in the three superinfected individuals relative to matched non-superinfected controls [13]. However in this same study, autologous pre-superinfection Envs were tested for neutralization only cross-sectionally against contemporaneous pre-superinfection and post-superinfection plasma, and heterologous breadth assays were performed against only two lab-adapted subtype B strains. Moreover, there was no evaluation of cross-neutralization of the superinfecting virus using plasma prior to superinfection [13]. Nevertheless, there is a common observation that superinfection occurred during the first year of infection, and was associated with low autologous neutralizing antibody responses [13]. These results are consistent with the hypothesis that higher susceptibility to superinfection during early infection may be, in part, due to diminished early humoral responses.

A different conclusion was reached from a study of superinfection in HIV-1

infected commercial sex workers in Mombasa, Kenya [10]. There it was shown that while NAb breadth and potency were lower in superinfected individuals than in matched controls after approximately one year of infection, no difference in these parameters occurred immediately prior to superinfection (between 0.72-5 years post-infection) [10]. In 4/6 cases identified in that study, superinfection occurred at or after two years of the initial infection, potentially allowing for development of stronger, yet still not protective, NAb responses [10]. Thus in this multiple HIV-1 subtype sex worker cohort, NAb did not appear to provide any protection from superinfection. While the authors did not investigate autologous NAb responses to transmitted/founder Env glycoproteins in the study, responses to initial variants cloned from the time of superinfection detection and early Envs from within the first year of infection were evaluated [10].

To evaluate cross-neutralization breadth prior to superinfection, we evaluated the potential of pre-superinfection plasma to neutralize not only superinfecting variants, isolated at the time superinfection was detected, but also a subtype C reference panel of pseudoviruses. We found that pre-superinfection plasma was unable to neutralize superinfecting variants and had limited ability to cross-neutralize a panel of variants prior to superinfection, with a range of 0–7 (of 12) variants neutralized at very low IC50s (20–70) amongst all three superinfected cases. Heterologous breadth in non-superinfected control plasma samples was similarly limited, though some individuals did have greater breadth but not potency. These data are consistent with previous studies, which showed that early autologous NAbs in subtype C infection are monotypic with limited cross-neutralization potential [22,23,26,31,32]. Furthermore, it has been demonstrated that significant cross-neutralizing antibody breadth is unlikely to occur prior to chronic infection [33,34].

Heterologous neutralizing antibody breadth did not necessarily correlate with

strength or effectiveness of autologous NAb responses. Although some non-superinfected individuals clearly mount strong autologous responses, they may exhibit limited neutralizing breadth by primarily targeting single or nonconserved epitopes [22,23,25,26,31,32,35,36]. In contrast, others with relatively low-titer autologous responses may in fact have wider breadth to multiple epitopes (or different epitopes), none of which confers a particularly effective neutralizing antibody response to the established infecting variant. Thus, this study suggests that, in the context of intrasubtype superinfections, either the ability to potently neutralize autologous virus or to target multiple epitopes could provide protection against superinfection. However, in the absence of both of these humoral responses, individuals may be predisposed towards superinfection.

Based on the data suggesting early deficits in NAb responses in superinfected individuals, but with little evidence for broadly neutralizing antibodies in the matched controls, we investigated whether levels of non-neutralizing antibodies also differed in the two groups prior to superinfection. We observed that superinfected individuals trended towards having lower levels of gp120-specific IgG antibodies prior to superinfection compared to controls, although this comparison did not achieve statistical significance ($p = 0.115$).

Similarly, we observed no reactivity to either consensus clade C or caseA2clB (clade B) V1V2-loop fusion proteins [8,30] in plasma from superinfected individuals prior to superinfection. By contrast in 3/10 non-superinfected matched controls, we observed reactivity to both proteins during the first 6 months, and in 6/10 controls reactivity was seen against the consensus C protein during the first year of infection. Higher levels of IgG V1V2-loop binding antibodies have been correlated with protection from primary HIV-1 infection in vaccinees that remained uninfected in the RV144 trial

[8,9], and the data presented here are consistent with the concept that these antibodies may contribute toward protection in individuals that remained only singly-infected.

In the RV144 trial, levels of IgA antibodies capable of binding to gp120 were directly correlated with the risk of infection [8,9]. It is of interest, therefore, that two of the three superinfected individuals showed the highest anti-gp120 plasma IgA levels amongst all study participants, while only two of the ten matched controls demonstrated positive IgA binding titers. One superinfected individual, ZM211F, showed no evidence of anti-gp120 IgA reactivity. However, this is consistent with the low overall HIV-1 specific humoral responses observed, including the lowest levels of V1V2-loop and gp120-specific IgG binding antibodies prior to superinfection. We have also found a statistically significant difference in anti-gp120 plasma IgA levels with respect to sexual exposure and potential HIV-1 acquisition risk, in that individuals either with superinfection (as a result of outside partnerships) or self-reported outside partnerships (in non-superinfected individuals) had significantly higher anti-gp120 plasma IgA responses ($p = 0.005$), as compared to non-superinfected controls without self-reported outside partnerships. This data corroborates those drawn from the RV144 trial that high plasma IgA levels may be a surrogate of HIV-1 exposure or a potential correlate of risk in the context of primary HIV-1 infection [8] and superinfection. We have yet to evaluate the mechanism by which these differences in plasma IgA levels may affect susceptibility to infection, however it has been suggested that high levels of IgA may interfere with other potentially protective antibody-mediated effector functions such as antibody-dependent cellular cytotoxicity (ADCC) [8]. Non-neutralizing IgG antibodies could play a major role in increased mucosal barrier protection, sequestering the virus at the epithelial surface and in female genital tract mucus and contributing to Fc receptor-mediated antiviral activity [6,37,38]. Thus a diminished non-neutralizing IgG antibody response,

compounded by potentially interfering IgA antibodies, could lead to reduced mucosal protection and higher susceptibility to superinfection. Future studies will elucidate whether non-neutralizing antibody-mediated antiviral activities contribute to protection from superinfection.

Conclusions

Our previous study demonstrated three intrasubtype C superinfections during the first year of infection, with no evidence of superinfection beyond year one in 19 individuals, despite longitudinal follow-up for more than three years [19]. This observation suggested that the risk of superinfection may be highest during the first year of infection, as has been predicted by mathematical modeling in a subtype B cohort [16]. Here we evaluated the potential of the humoral immune response in natural infection to protect against superinfection. Autologous NAb responses were markedly delayed and lower in magnitude in superinfected individuals prior to superinfection detection ($p = 0.039$). Because of the strain-specific nature of early autologous NAb, this difference in titers may be a surrogate marker for a potential immunological deficit in protective antibodies or another factor contributing to effective humoral responses. Nevertheless, if confirmed in a larger ongoing study, these data provide support for the feasibility of inducing a protective immune response via an HIV-1 vaccine, in regions where subtype diversity is limited. It will be critical to understand the nature of vaccine-induced humoral responses and to what degree these antibodies can protect from *de novo* infection.

Acknowledgements

We gratefully recognize all of the RZHRG (Rwanda Zambia HIV Research Group) and ZEHRP (Zambia-Emory HIV Research Project) staff and the participants who made this study possible. We would also like to acknowledge Dr. Jesus Salazar (University of Alabama Birmingham) for contributing the ZM247Fv1-fs env clone and Dr. Chavdar Krachmarov, Kathy Revesz and William J. Honnen (Public Health Research Institute at the University of Medicine and Dentistry of New Jersey) for P623 (MuLVgp70-caseA2clB V1V2), P621 (MuLV gp70 carrier control) and P2442 (MuLVgp70-conC V1V2) proteins. We obtained the Subtype C HIV-1 Reference Panel of Env Clones (cat # 11326) through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

This research was supported by grants from the National Institutes of Health (R37 AI-51235 (EH); R01 AI-58706 (CAD); U01 AI-78410 (AP), R56 AI091514-01A1 (PTH)) and the International AIDS Vaccine Initiative (SA). A Fogarty AITRP grant FIC 2D43 TW001042 supported WK. The Virology Core at the Emory Center for AIDS Research supported viral load testing (P30 AI050409). CSK was supported by an NRSA Institutional Postdoctoral Training Grant (T32 AI-007470) and National Institutes of Health/National Center for Research Resources (KL2 RR-025009). This project was also funded in part by the National Center for Research Resources P51RR165 and is currently supported by the Office of Research Infrastructure Programs/OD P51OD11132.

Nucleotide sequences: GenBank SGA accession numbers for ZM247F: EU166821.1, EU166792.1, EU166799.1, EU166800.1, EU166802.1, EU166816.1, EU166819.1. GenBank accession numbers for longitudinal SGA from superinfected individuals [19]: JX239263 -

JX239437. GenBank accession numbers for full-length founder/early *env* SGA in this study: JX213352 - JX213473.

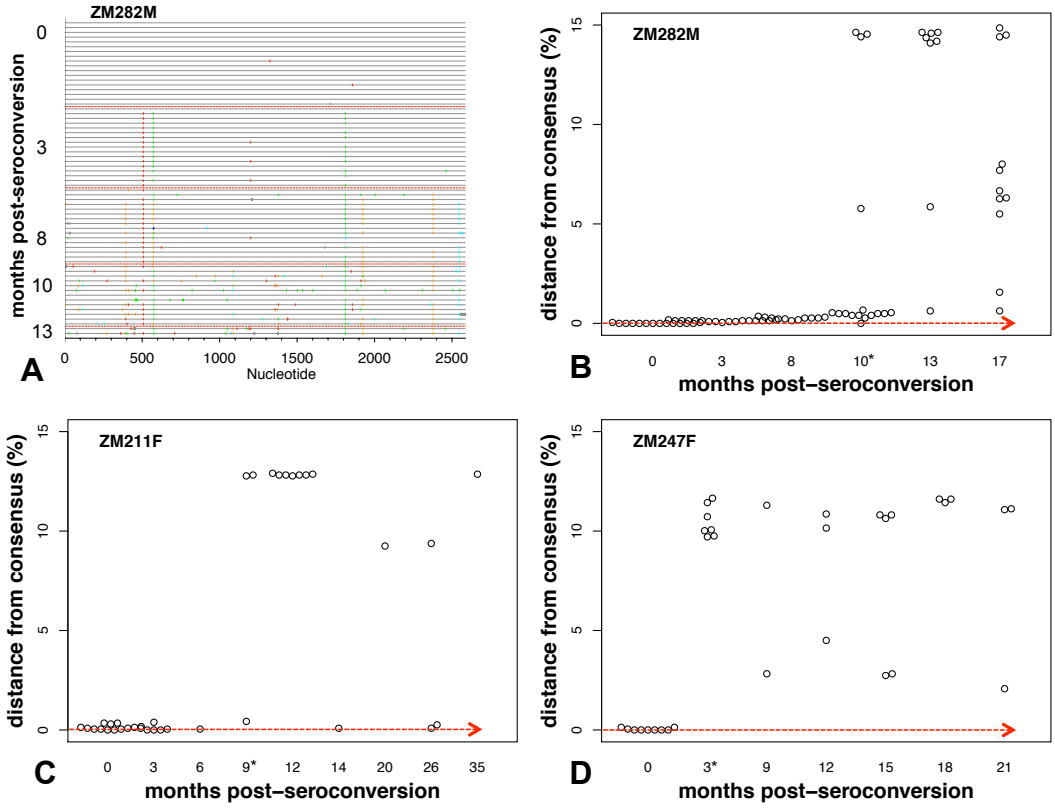

Figure 1. Homogeneity of early/founder env sequences prior to superinfection in three intrasubtype C superinfected individuals. Single genome amplified full-length *env* sequences were evaluated through Highlighter plots for visualization of viral evolution of early/founder variants. Nucleotide changes from the early/founder consensus *env* sequence can be visualized by the colored hatch-marks (red=T, green=A, orange=G, light blue=C) as shown with ZM282M **(A)**, in which superinfection was detected at 10 months post-seroconversion (superinfecting sequences not shown). The time point from which amplicons were isolated is shown as months after seroconversion (0-month). Raw pairwise distance from the early/founder consensus sequence to each longitudinal *env* amplicon sequence (vertical axis) was plotted for the three superinfected cases **(B-D)**. Asterisks on the x-axis indicate time at which superinfection was detected and

superinfecting sequences are included, clustering at 9-15% pairwise distance from initial consensus **(B-D)**.

Table 1. Seroconverters from ZEHRP cohort evaluated for longitudinal autologous neutralization of initial variants

Subject ID	Linkage	Subtype	Last Seronegative Date	First Sample Date	Estimated days from infection[a]	Viral load[b]	Setpoint Viral Load[c]	Estimated timing of SI[d]	Viral load at SI[e]	Sex with Condom[f]	Sex without Condom[f]	Outside Partner[g]	Initial Virus Env[h]
ZM282M	**Unlinked**	**C**	**8-Dec-04**	**3-Mar-05**	**43**	**>750,000**	**253,000**	**10**	**300,000**	**78**	**2**	**Yes**	**Founder**
ZM211F	**Unlinked**	**C**	**15-Mar-02**	**5-Jul-02**	**60**	**2640**	**14, 454**	**9**	**14,454**	**11**	**3**	**No**	**Early**
ZM247F	**Unlinked**	**C**	**29-Jul-03**	**1-Nov-03**	**26**	**>750,000**	**67,472**	**3**	**43,428**	**112**	**26**	**No**	**Founder**
ZM1072M	Unlinked	C	19-May-06	16-Aug-06	45	36,200	15,400	N/A	N/A	144	0	No	Founder
ZM297M	Unlinked	C	4-Mar-05	9-Jun-05	49	8,687	45,500	N/A	N/A	108	3	No	Early
ZM1464M	Unlinked	C	9-Dec-06	10-Mar-07	46	44,500	15,600	N/A	N/A	68	0	No	Early
ZM503F	Unlinked	C	2-Nov-06	9-Feb-07	50	25,800	17,100	N/A	N/A	65	0	No	Founder
ZM249M	**Unlinked**	**C**	**6-May-03**	**12-Aug-03**	**29**	**>750,000**	**82,233**	**N/A**	**N/A**	**145**	**25**	**Yes**	**Founder**
ZM267F	Unlinked	C	23-Mar-04	29-Jun-04	29	>750,000	50,061	N/A	N/A	188	8	No	Founder
ZM284M	Unlinked	C	14-Jan-05	9-Apr-05	22	>750,000	354,880	N/A	N/A	97	1	No	Early
ZM289M	Unlinked	C	30-Apr-05	19-May-05	41	399,737	4,512	N/A	N/A	75	8	No	Founder
ZM237M	Unlinked	C	29-Apr-03	29-Jul-03	46	44,870	59,250	N/A	N/A	41	4	No	Early
ZM184F	**Unlinked**	**C**	**11-Apr-03**	**10-Jul-03**	**45**	**71,290**	**72,718**	**N/A**	**N/A**	**39**	**9**	**Yes**	**Early**

Bolded subjects: superinfected individuals with evidence of superinfection from outsider partnerships or non-superinfected controls who self-reported outside partnerships

[a]Days from time of infection to first sample date was calculated, as follows: If only first Ab+ dates were available, time of infection was determined by the midpoint between last seronegative and first Ab+ date. If Ag+Ab- dates were available, time of infection was estimated by subtracting 22 days from Ag+Ab- dates. (Haaland 2009)

[b]Viral load: RNA copies/ml in plasma at first sample date

[c]Viral load: RNA copies/ml in plasma at 7-12 months post-seroconversion

[d]Months post-seroconversion in which superinfection was detected

[e]Viral load: RNA copies/ml in plasma at time of superinfection detection

[f]Sexual data is cumulative events for 12 months (Kraft 2012)

[g]Other partners self-reported for 12 months (Kraft 2012)

[h]Denotes whether initial virus Env tested represents a transmitted/founder Env or an early founder-like Env isolated from first sample date

Abbreviations: superinfection (SI), Envelope (Env), Not Applicable (N/A)

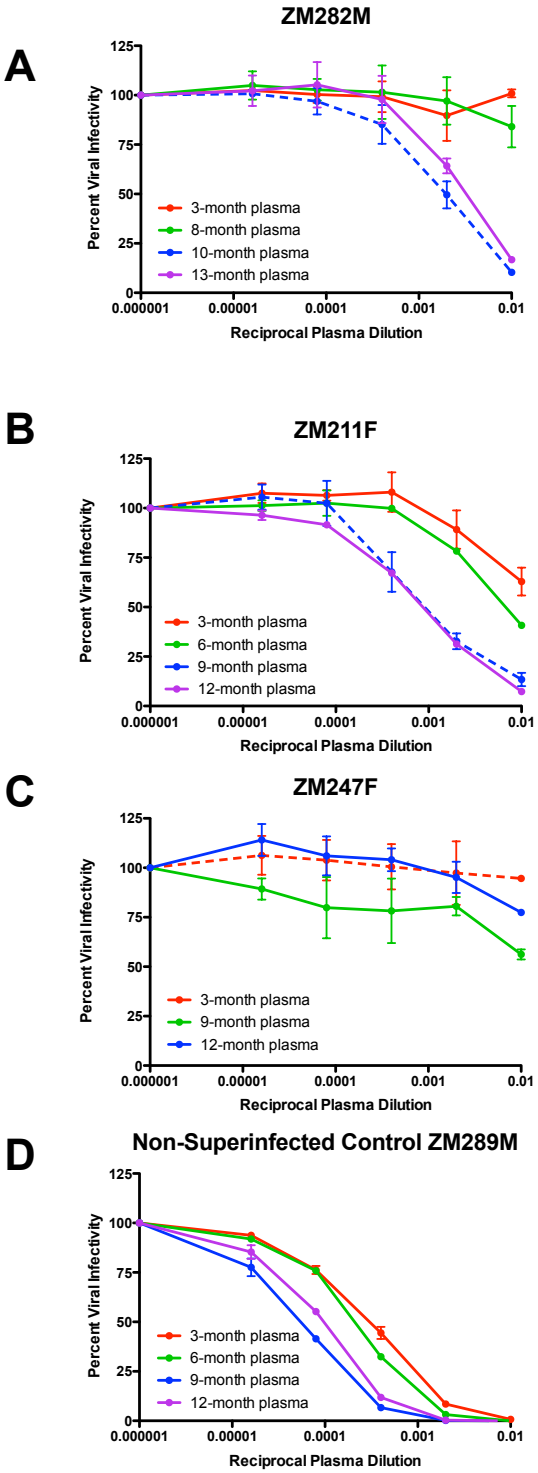

Figure 2. Autologous neutralizing antibody responses to early/founder Env in superinfected individuals during early infection. Early/founder viruses were tested for

neutralization by autologous plasma from the first year of infection in superinfected **(A-C)** and non-superinfected controls (representative control shown in panel **D**). Dashed lines correspond to plasma from the time point at which superinfection was detected. Percent viral infectivity is depicted on the vertical axis, and reciprocal plasma dilution is depicted along the horizontal axis, in logarithmic fashion. Each curve represents a single plasma-virus combination, performed in duplicate wells. Error bars represent standard error of the mean between two independent experiments.

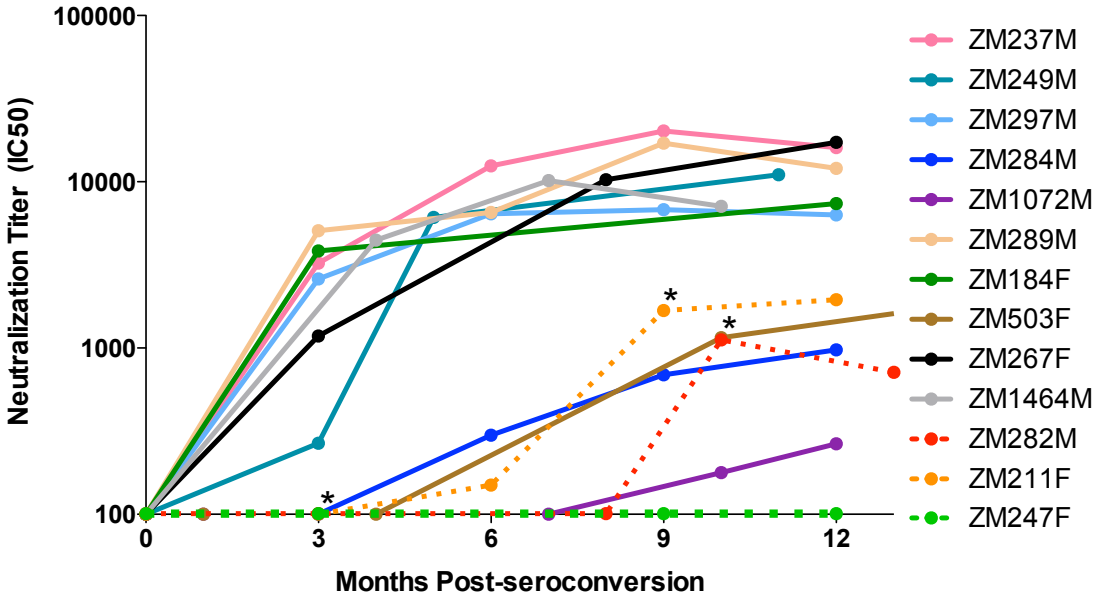

Figure 3. Development of autologous neutralizing antibodies to early/founder virus Env is slow or absent prior to superinfection. Plasma neutralizing antibody IC50 titers (representing plasma dilution necessary to achieve 50% viral infectivity) to early/founder virus Env were determined over the course of the first year of infection for three superinfected (dashed lines) and ten non-superinfected matched controls (solid lines). Values represent mean IC50 values from two independent experiments. Asterisks mark time at which superinfection was detected in the superinfected cases.

A.

Founder Env Pseudovirus	1st plasma time point	2nd plasma time point	3rd plasma time point	4th plasma time point
	1-4 month	5 - 8 month	9-10 month	11-15 month
ZM282M – SI	**<100**	**<100**	**1120***	**713**
ZM211F – SI	**<100**	**150**	**1683***	**1950**
ZM247F – SI	**<100***		**<100**	**<100**
ZM1072M – Non-SI	<100	<100	178	266
ZM297M – Non-SI	2609	6413	6799	6329
ZM1464M – Non-SI	4464	10185	7133	
ZM503F – Non-SI	<100		1157	2011
ZM249M – Non-SI	**268**	**6114**		**11037**
ZM267F – Non-SI	1183	10286		17297
ZM284M – Non-SI	<100	299	690	976
ZM289M – Non-SI	5083	6540	17090	12045
ZM237M – Non-SI	3238	12490	20240	16034
ZM184F – Non-SI	**3840**			**7404**

B.

Superinfecting Env Pseudovirus	1st plasma time point	2nd plasma time point	3rd plasma time point	4th plasma time point
	3 month	5 - 8 month	9-10 month	11-13 month
ZM282M – SI	<100	<100	<100 *	<100
ZM211F – SI	<100	<100	<100 *	<100
ZM247F – SI	<100 *		282	1865

IC50 value key	<100	100-1000	1001 -10,000	10,000+

Figure 4. Summary of neutralization titers to initial and superinfecting variants.

Plasma neutralizing antibody IC50 titers to the transmitted founder virus Env were determined over the course of the first year of infection for three superinfected (ZM282M, ZM211F and ZM247F, bolded) and ten non-superinfected (non-SI) case-matched controls (A). Non-superinfected controls that had self-reported outside partnerships are also bolded. Similarly, IC50s to superinfecting variants were determined over the course of the first year of infection for all three superinfected cases (B). Values represent mean IC50 values from two independent experiments. Asterisks mark time in which superinfection was detected in the superinfected cases.

		1b	1b	2	2	2	2	2	2	2	2	2	2		
Subject ID	Plasma Month	ZM197M.PB7	ZM109F.PB4	Du156.12	Du172.17	Du422.1	ZM214M.PL15	ZM233M.PB6	ZM249M.PL1	ZM53M.PB12	ZM135M.PL10a	CAP45.2.00.G3	CAP210.2.00.E8	Breadth Score[a]	Potency Score[b]
ZM282M - SI	**8**	**30**	**70**	**70**	**30**	**10**	**10**	**50**	**30**	**10**	**10**	**35**	**10**	**7**	**29**
ZM211F - SI	**6**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**0**	**10**
ZM247F - SI	**3**	**10**	**35**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**20**	**10**	**10**	**2**	**11**
ZM1072M – Non-SI	7	60	35	10	30	10	20	50	10	10	35	35	20	**8**	**24**
ZM297M – Non-SI	6	30	10	10	10	10	10	10	10	10	10	10	10	**1**	**11**
ZM1464M – Non-SI	7	35	20	30	10	10	35	10	10	10	40	10	10	**5**	**17**
ZM503F – Non-SI	10	80	50	80	20	10	55	10	20	10	35	35	35	**9**	**33**
ZM249M – Non-SI	**5**	**95**	**55**	**10**	**10**	**10**	**10**	**10**	*auto	**10**	**10**	**10**	**10**	**2**	**15**
ZM267F – Non-SI	8	315	60	200	60	10	35	75	30	10	70	20	10	**9**	**59**
ZM284M – Non-SI	6	165	50	130	90	45	100	70	60	40	35	40	75	**12**	**73**
ZM289M – Non-SI	6	10	10	10	10	10	10	10	10	10	10	10	10	**0**	**10**
ZM237M – Non-SI	6	10	40	10	10	10	10	10	10	10	10	10	10	**1**	**11**
ZM184F – Non-SI	**12**	**440**	**50**	**10**	**100**	**25**	**10**	**180**	**80**	**10**	**60**	**55**	**90**	**9**	**73**

IC50 value key	<20	20-50	51-100	100+

Figure 5. Cross-neutralizing Breadth and Potency against HIV-1 Subtype C Env Reference Panel. Bolded individuals represent superinfected individuals with evidence of superinfection from outsider partnerships (ZM282M, ZM211F and ZM247F) or non-superinfected controls who self-reported outside partnerships. Pre-superinfection plasma for superinfected individuals or similar plasma time points for non-superinfected controls was tested against a panel of twelve Subtype C envelope pseudoviruses. This panel included Envs of both Tier 1b and Tier 2 sensitivities. Starting plasma dilution was reduced to 1:20 to increase assay sensitivity. Plasma-env combinations, which did not reach an IC50 value at the lowest dilution tested (1:20), were assigned a value of 10. Breadth score was calculated by adding the total number of envelopes neutralized at an IC50 greater than or equal to 20. Potency score was calculated by dividing individual plasma-env IC50 by median IC50 per envelope against all plasma and then adding the sum of these scores (rounded to the nearest integer) for each plasma. “Auto” indicates that a plasma sample was tested against an autologous envelope in the panel, IC50 values were not counted in breadth and potency scores.

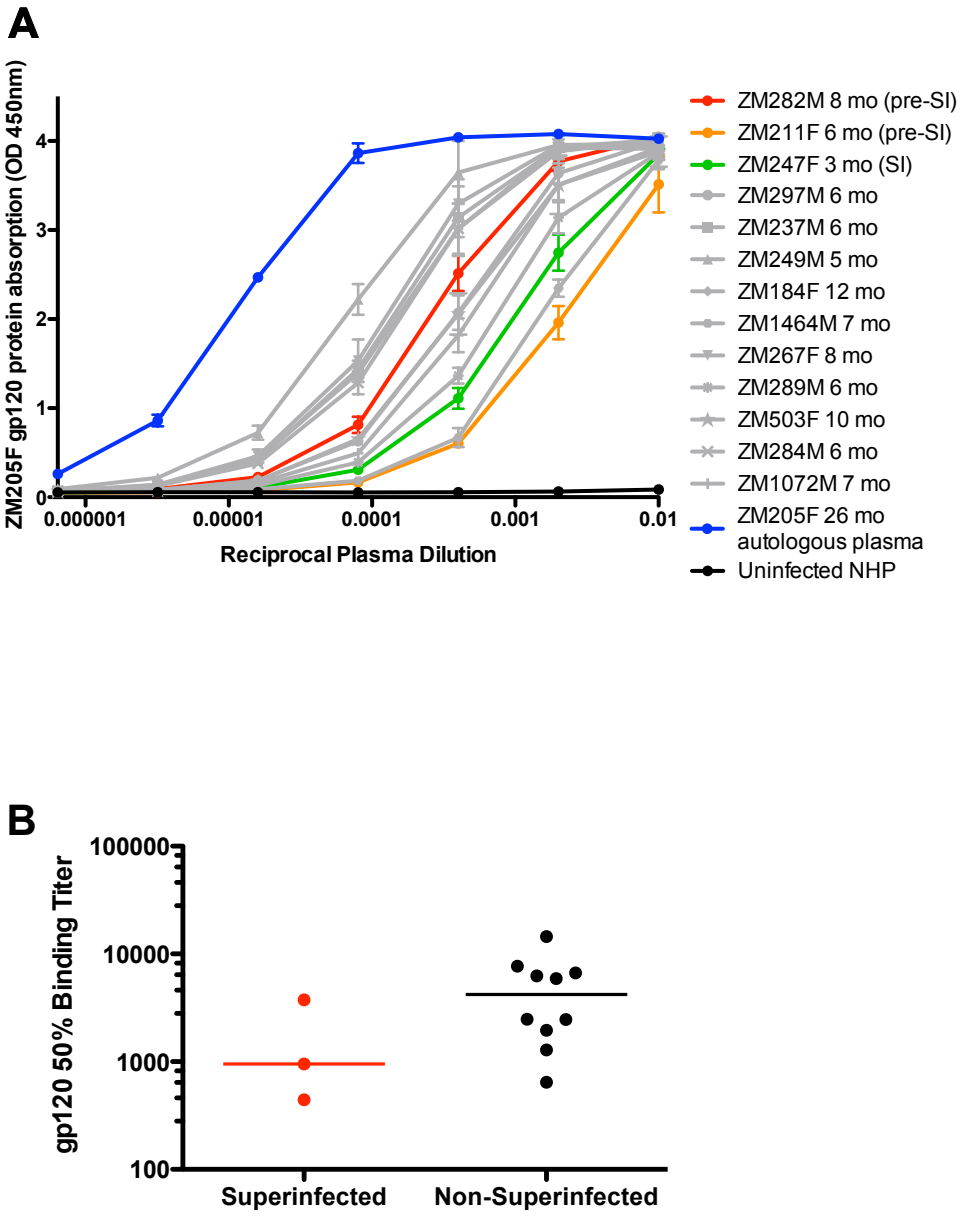

Figure 6. Plasma IgG binding antibody levels to purified subtype C gp120 protein is also reduced in superinfected individuals. Purified gp120 protein from the Zambian subtype C seroconverter ZM205F [23, 44] was used with serial dilutions of plasma in a gp120 binding ELISA. Autologous plasma from ZM205F was used as a positive control for presence of gp120-specific binding antibodies (blue). Levels of gp120-specific IgG binding antibodies in plasma from time points prior to superinfection for superinfected

individuals (ZM282M: red, ZM211F: orange) and similar plasma time points for non-superinfected controls (grey) was measured as shown in panel **A**. For ZM247F, in which superinfection was detected at 3-months post-seroconversion, we tested this 3-month plasma (green). Values for 50% gp120 binding in this assay were determined and compared between superinfected and non-superinfected groups **(B)** using both a mixed-linear effects model (p=0.115) and a Mann-Whitney test to compare medians between the groups (p=0.161).

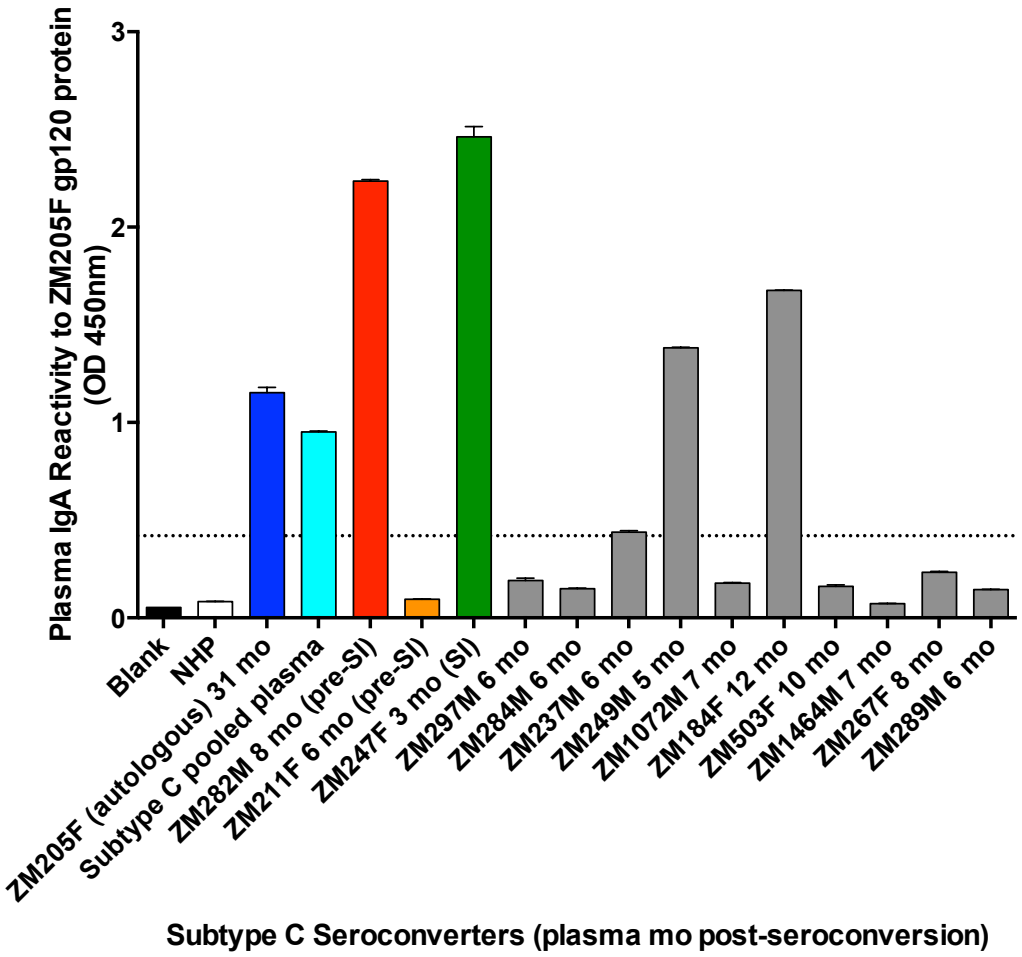

Figure 7. Plasma IgA levels to purified subtype C gp120 protein are highest in two of the three superinfected individuals. Purified gp120 protein from the Zambian subtype C seroconverter ZM205F [23, 44] was used with serial dilutions of IgG-depleted plasma in a gp120 binding ELISA. Autologous plasma from ZM205F was used as a positive control for presence of gp120-specific binding antibodies (dark blue). Levels of gp120-specific IgA binding antibodies in IgG-depleted plasma from time points prior to superinfection for superinfected individuals (ZM282M: red, ZM211F: orange) and similar plasma time points for non-superinfected controls (grey) was measured at a 1:125 plasma dilution. For ZM247F, in which superinfection was detected at 3-months post-seroconversion, we tested this 3-month plasma (green). Positive absorption was recognized as absorption

greater than five-times that of the normal human plasma (NHP) control and is shown as a dashed line.

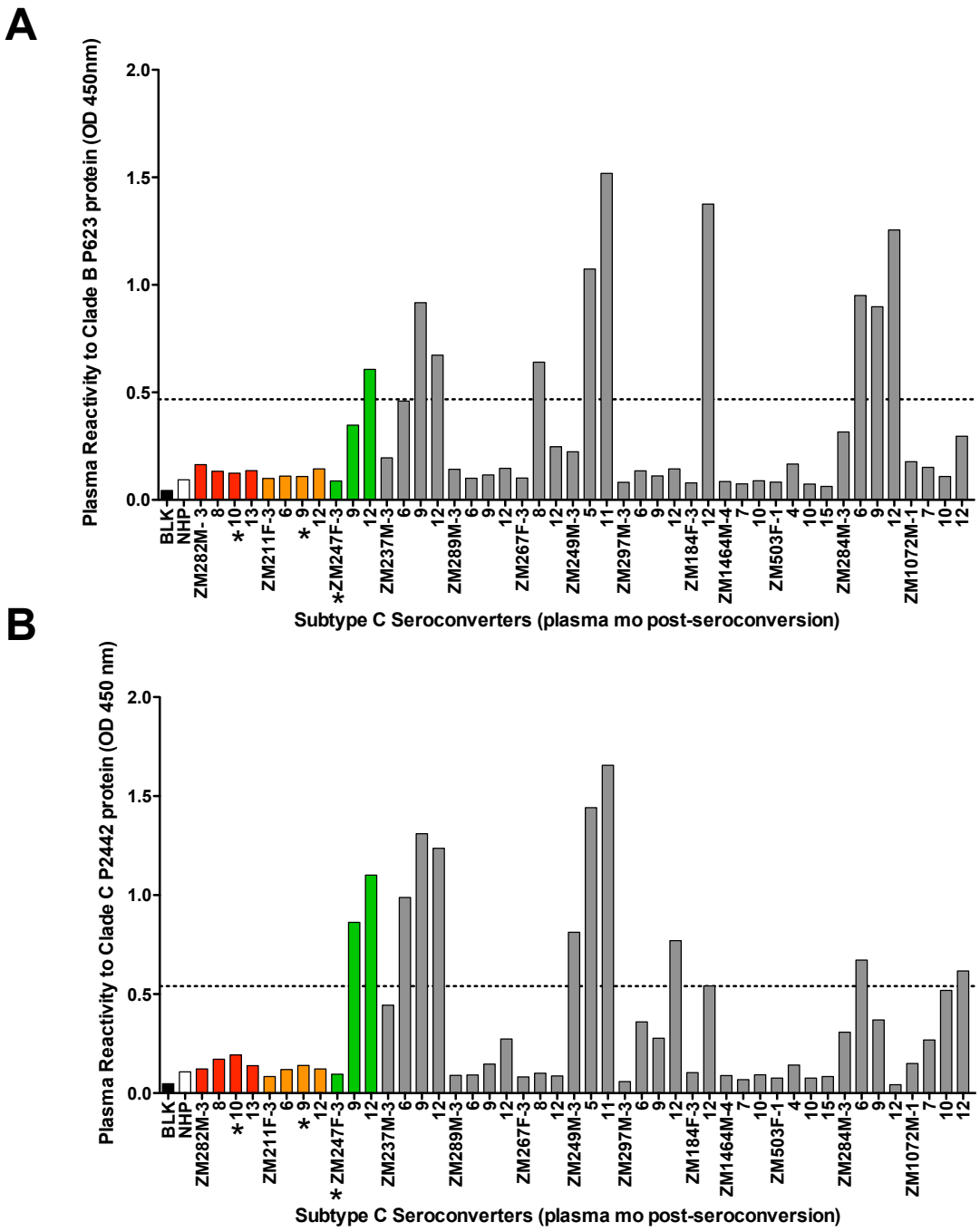

Figure 8. Plasma binding antibodies to both clade B and C gp120 V1V2-loop proteins are absent in superinfected individuals prior to superinfection. Plasma reactivity (at a single 1:500 dilution) to both P623 MuLVgp70-caseA2clBV1V2 [29] **(A)** and P2442 MuLVgp70-consensus clade C V1V2 **(B)** proteins was measured in a standard ELISA

assay. Longitudinal plasma from the first year of infection in both superinfected (colored) and non-superinfected (grey) controls was tested. Asterisks denote time at which superinfection was detected. Positive absorption was recognized as absorption greater than five-times that of the normal human plasma (NHP) control and is shown as a dashed line. Figure is representative of two independent experiments.

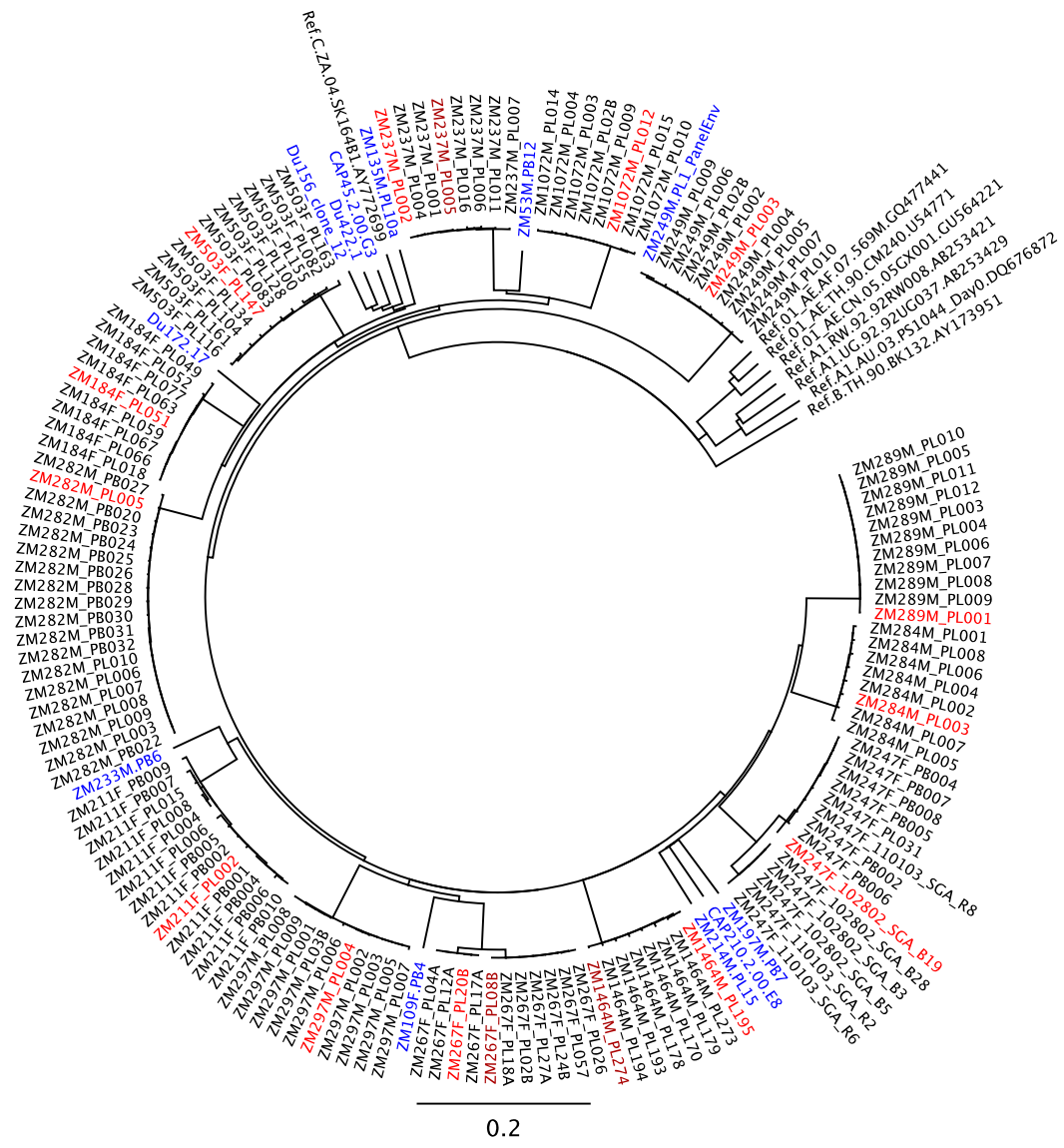

Figure S1. Radial neighbor-joining phylogenetic tree of full-length env amplicon sequences. This phylogenetic tree shows sequences of all full-length gp160 *env* amplicons isolated from time of seroconversion evaluated in this study in order to infer the subtype C early/founder Envs tested (red). Additional amplicons tested are shown in maroon, though no functional difference in neutralization phenotype between amplicons of the same patient was seen (data not shown). Sequences of Envs from the Subtype C HIV-1 Reference Panel of Env Clones panel [27, 28] are also shown (blue).

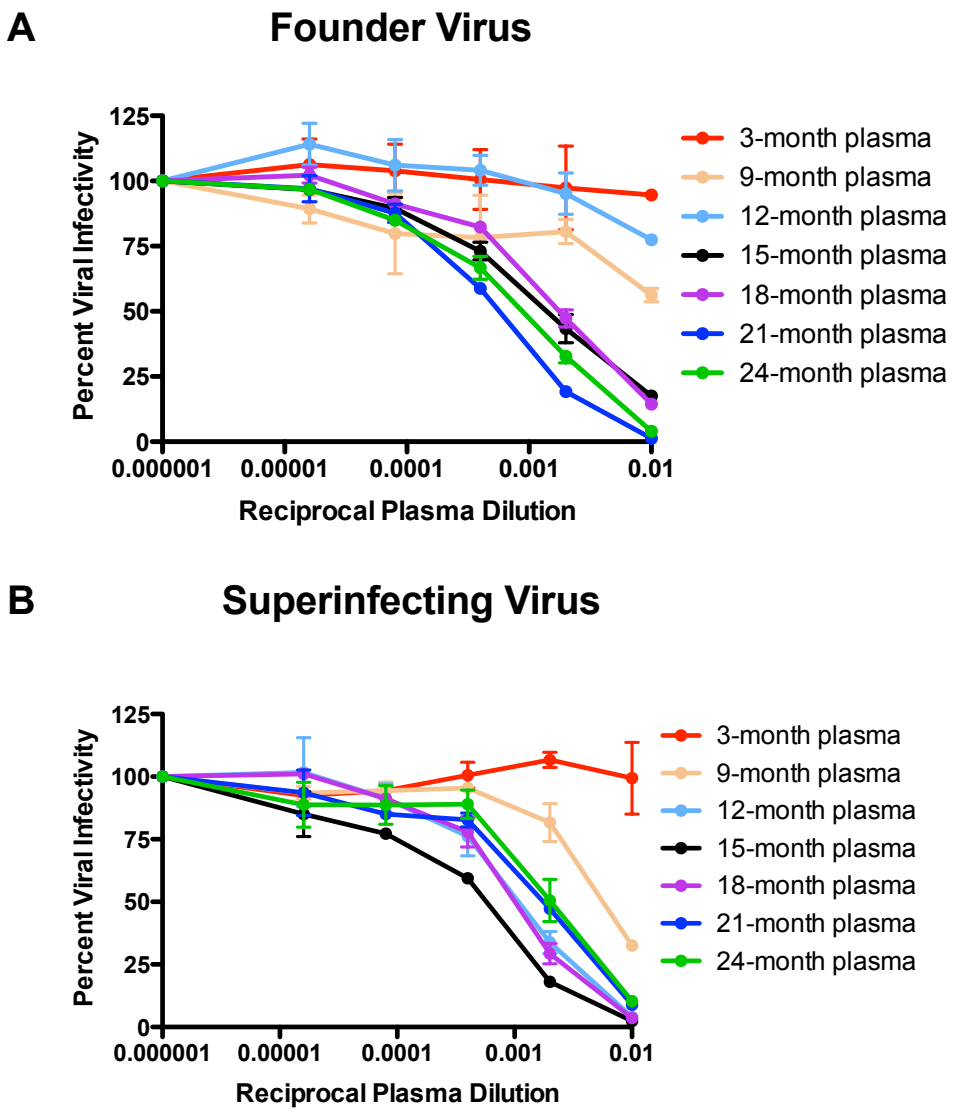

Figure S2. Preferential neutralization of superinfecting virus Env is observed in one case of early intrasubtype C superinfection. Autologous neutralizing antibody responses to both founder Env **(A)** and superinfecting Env **(B)** were measured over the first two years for ZM247F, in which superinfection was detected at 3-months post-seroconversion. Percent viral infectivity is depicted on the vertical axis and reciprocal plasma dilution is depicted along the horizontal axis, in logarithmic fashion. Each curve

represents a single plasma-virus combination, performed in duplicate wells. Error bars represent standard error of the mean between two independent experiments.

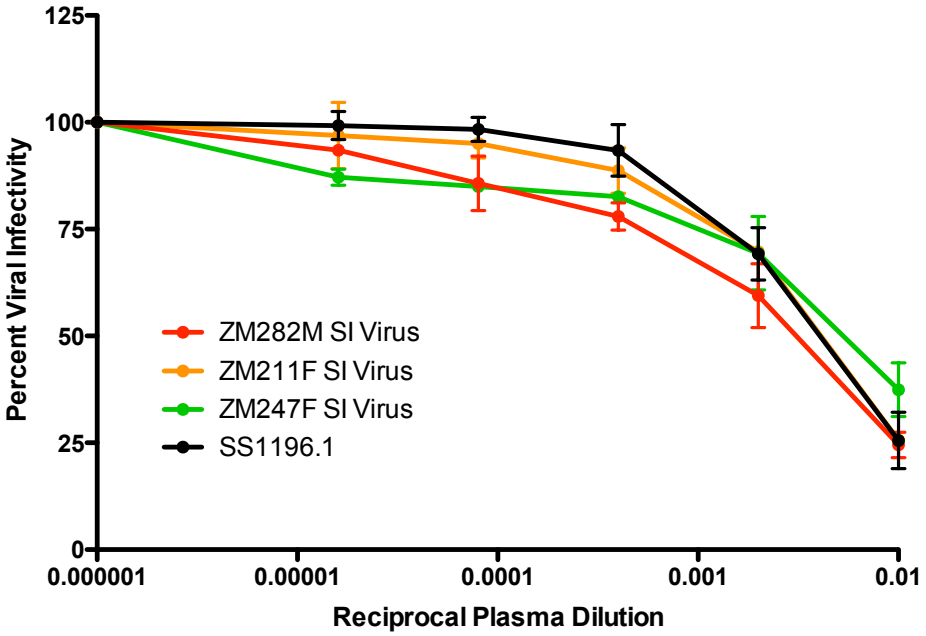

Figure S3. Superinfecting viruses are sensitive to neutralization by pooled subtype C plasma. We tested the ability of pooled subtype C plasma to neutralize superinfecting pseudoviruses from all three superinfected cases, in addition to SS1196.1 pseudovirus (carrying an envelope with Tier1b sensitivity) for comparison [27]. Percent viral infectivity is depicted on the vertical axis and reciprocal plasma dilution is depicted along the horizontal axis, in logarithmic fashion. Each curve represents a single plasma-virus combination, performed in duplicate wells. Error bars represent standard error of the mean between two independent experiments.

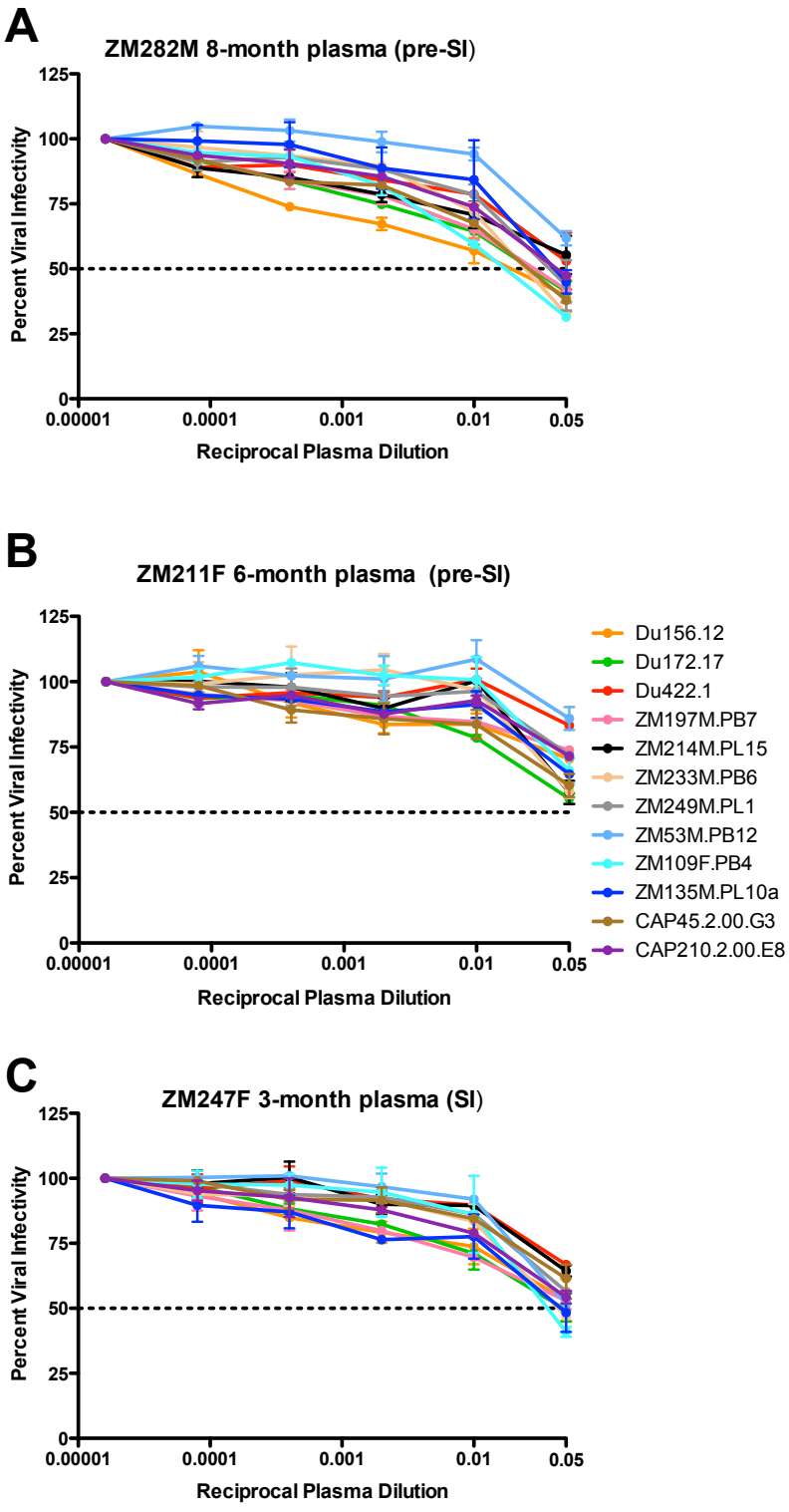

Figure S4. Limited heterologous neutralizing antibody breadth in superinfected individuals prior to superinfection. Plasma from pre-superinfection (**A, B**) or early

superinfection (SI), in the case of ZM247F **(C)**, time points was tested for heterologous neutralization to a subtype C Env reference panel. This panel included Envs of both Tier 1b and Tier 2 sensitivities [27, 28]. Starting plasma dilution was reduced to 1:20 to increase assay sensitivity. Percent viral infectivity is depicted on the vertical axis and reciprocal plasma dilution is depicted along the horizontal axis, in logarithmic fashion. Each curve represents a single plasma-virus combination, performed in duplicate wells. Error bars represent standard error of the mean between two independent experiments.

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Chapter 4: Discussion

Hope for an HIV-1 Vaccine

To date, over 187 clinical trials have been initiated to test potential HIV-1 vaccine immunogens and identify promising candidates for relevant human vaccine efficacy trials and subsequent licensure of a global HIV-1 vaccine [200]. The ultimate goal of an HIV-1 vaccine would be one that confers sterilizing immunity (complete protection from HIV-1 infection). However, another strategy would be to elicit vaccine-induced control of viremia and subsequent immunopathology, limiting establishment of a latent reservoir and the potential for transmission to a new host [201]. These options are not mutually exclusive, though, and a truly successful HIV-1 vaccine will likely induce both protective antibodies and cellular immunity capable of controlling viremia in the event of incomplete sterilizing immunity.

For a vaccine to be licensed, however, reliable and consistent results demonstrating vaccine-elicited correlates of protection must be shown [202]. The correlates of protection from HIV-1 infection have yet to be firmly identified despite numerous approaches, including passive transfer studies in animal models, evaluation of individuals with apparent resistance to HIV-1 acquisition (highly exposed but seronegative individuals, individuals with delta32 CCR5 polymorphism, etc.), consideration of immune factors affecting HIV-1 superinfection and of course, evaluation of immune responses in individuals vaccinated with different HIV-1 vaccine immunogens under various vaccination regimens. This discussion will cover the lessons learned about the correlates of protection from major phase IIb and III vaccine trials. In addition, it will focus on superinfection studies, which have provided another natural model by which to consider primary immune responses affecting susceptibility to and potential protection from re-infection.

Although the correlates of HIV-1 protection have yet to be concretely defined, there are some things that are known: 1) all licensed vaccines for infectious diseases rely to some degree on neutralizing antibodies as a correlate of protective immunity [87], 2) passive antibody transfer studies have supported the idea that neutralizing antibodies can protect from acquisition in animal models of HIV/SHIV infection [203], 3) historically, HIV-1 vaccines that have solely sought to elicit CD8+ T cell mediated immunity have not been protective against HIV-1 acquisition [204], 4) recent evidence of vaccine-mediated humoral immunity (through Env-specific non-neutralizing antibodies) has been identified in the immune correlates analysis of the RV144 vaccine efficacy trial [93], 5) broadly neutralizing antibodies (bnAbs) have been identified which can neutralize heterologous primary isolates from multiple subtypes (though these bnAbs are rare and as of yet, have not been easily elicited) [205], and finally, 6) in some cases, individuals who become superinfected with HIV-1 lack potent titers of protective antibodies prior to superinfection, again implicating antibodies as a potential direct correlate or indirect surrogate marker of protection [177, 185]. Despite the fact that cellular immunity via CD8+ T cells will undoubtedly need to be elicited for viral control in the event of breakthrough infections, these aforementioned facts support the notion that vaccine-mediated protection from HIV-1 acquisition may be possible through Env-specific antibodies. However, pinpointing what immunogens may best elicit this protection is one of the major questions facing the field of HIV vaccinology.

Many of the early vaccine trials focused on Env immunogens (in the form of monomeric gp120 glycoprotein or the gp160 Env precursor) isolated from early lab-adapted HIV-1 strains (e.g. IIIB, MN, SF2) rather than primary HIV-1 isolates [201]. Env was initially pursued as a putative vaccine immunogen since it was shown in passive transfer studies that neutralizing antibodies and cross-reactive monoclonal antibodies to Env could confer protection in the setting of chimeric simian-HIV (SHIV) or HIV-1

challenge in animal models [82-91, 94-96]. However, although several of these Env immunogens (including the phase III bivalent Vax003 AIDSVAX subtype B/E gp120 in alum and phase III bivalent Vax004 AIDSVAX subtype B/B gp120 in alum vaccines) generated neutralizing antibodies to the vaccine strain (and in some cases other easy-to-neutralize Tier 1 viruses like SF162 and BaL), these Nabs did not appear to confer protection from infection with primary HIV-1 isolates or affect plasma viral load after infection [201, 206-208]. In addition, this vaccine-mediated Nab response was often short-lived with a half-life of around 8-weeks [201]. These trials suggested that the Nab responses elicited by these constructs were not useful for eliciting enduring vaccine-mediated cross-protection from primary HIV-1 isolates.

T-cell based vaccines have also resulted in major disappointment in human vaccine efficacy trials, despite showing promising efficacy in long-term non-human primate studies. A huge blow to the HIV vaccine field was the failure of the phase IIb Merck Step trial (HVTN 502) in 2008. The results showed not only a lack of protection from HIV-1 infection, but also an increased risk of HIV-1 acquisition in uncircumcised vaccinated men who had pre-existing immunity (Nab) to the vaccine vector construct of Adenovirus 5 (Ad5) [209]. This vaccine, which was composed of a recombinant Ad5 vector with Subtype B HIV-1 Gag, Pol and Nef, relied on eliciting protective CD8+ T-cell mediated immunity. Although it appears that cellular pressure was applied to the founder virus, as evident from immune escape mutations in breakthrough viruses [200, 210], the T cell response generated by this vaccine did not show any ability to reduce plasma viral load [209]. The Gag, Pol and Nef viral sequences that were chosen as the immunogens in this vaccine were based on native sequences from Subtype B primary isolates resembling subtype B consensus [204]. Vaccinees were high-risk MSM and heterosexual men and women largely in the Americas, Australia and the Caribbean. The Phambili trial (HVTN503), using the same Ad5-based vaccine as HVTN502, also failed

to show any protective efficacy or reduction in viral load in a population of heterosexual men and women from South Africa (regionally affected by Subtype C HIV-1). Similarly, follow-up analyses of this study also showed a slightly higher incidence of infection in vaccinees [211].

These studies gave little hope for the feasibility of an HIV-1 vaccine eliciting either Ab-based or CTL-based responses in isolation. Rather, an Env-based component will likely be required for the best protection from HIV-1 acquisition via protective antibodies, while a vaccine would also need a potent cellular immune response for viral control. Though this cell-mediated viral control has yet to be observed in human vaccine efficacy trials, replication-competent cytomegalovirus (CMV)-based vectors for SIV vaccination in Rhesus macaque models have shown a surprising early and durable control of SIV viremia in animals that later became infected, which was attributed to the presence of vaccine-induced effector memory T cells at sites of SIV replication [97, 204, 212].

In addition, whether cell-mediated immunity should target conserved regions of the virus that would result in a loss of fitness should it try to escape, or whether constructs should be designed to generate responses to a wider breadth of viral regions continues to be evaluated. A recent study by Barouch et al. showed that in the setting of Rhesus macaques vaccinated with a combination of adenovirus and poxvirus vectors expressing SIV antigens and multiply-challenged with heterologous $SIV_{mac}251$ virus, viral control was dependent on the magnitude and breadth of Gag-specific CD8+ T cell responses. However, protection from SIV acquisition was entirely dependent on Env, specifically in eliciting Env-binding and Tier 1 neutralizing antibodies [97, 204, 213].

These studies reiterate the idea that protection from HIV-1 infection will rely on the generation of Env-specific antibodies. Identifying the most relevant and effective Env immunogen to elicit protective antibody responses from a vaccine is still an area of

intense research, and protective vaccine-mediated humoral immunity has only recently been shown. Prior to 2009, the question of whether protective immunity could be generated from an HIV vaccine was a very real and disheartening concern. However in 2009, surprising results of a vaccine efficacy trial called RV144, which used two constructs previously shown to lack efficacy in isolation and in a different risk group of vaccinees (Thai IDU), demonstrated a moderate 31.2% reduction in risk from HIV-1 infection in relatively low-risk heterosexual Thai vaccinees versus placebo recipients [92]. While this protection was modest and extrapolation of results has been subject to harsh scrutiny, it was still the first vaccine efficacy trial to show any protection elicited from a vaccine regimen. This proof-of-concept has encouraged the field to push forward with identification of correlates of protection from the vaccine and optimization towards higher level of protection, with renewed hope that protection via vaccination may be possible.

The RV144 vaccine was a heterologous prime-boost regimen, comprised of a CD4+ stimulating recombinant canarypox vector (ALVAC) carrying HIV-1 gp120 (from a CRF01_AE isolate, TH023), Subtype B gag and protease prime with a bivalent Subtype B/E gp120 (AIDSVAX) protein boost. Although this vaccine did not elicit broadly neutralizing antibodies or potent CD8+ T cell responses, it did show stimulation of antigen-specific CD4+ T cells, neutralizing antibodies to Tier 1 (easy-to-neutralize) viruses and non-neutralizing antibody responses [97, 214, 215]. Immune correlates analyses comparing immune responses in vaccinees (41 that were subsequently infected and 205 that remained uninfected over the 3-year follow-up period) identified two potential immunological correlates of risk or protection from primary HIV-1 infection, both of which were associated with non-neutralizing antibodies [93, 97]. The first correlate was that vaccinees that remained protected from infection had higher levels of binding IgG antibodies to the V1V2 loops of Env. Follow-up analysis evaluating

breakthrough viral sequences has suggested that vaccine efficacy was partially related to antibody recognition of amino acid signatures in V2 [200, 210]. However, it is still unclear what specific mechanistic role these V2 antibodies were able to play in protection. Whether this finding was anecdotal or may be an important direct or indirect immune correlate of protection that should be elicited in an HIV vaccine remains to be determined. The second correlate was that vaccinees that became subsequently infected had higher levels of plasma IgA (specific to the C1 region of gp120) [93]. Furthermore, in individuals with low plasma IgA, ADCC and Nab responses seemed to correlate with decreased risk of infection, suggesting that the high levels of plasma IgA may antagonize potential IgG-mediated activity [93, 200]. This hypothesis was supported by results demonstrating that these C1-specific IgA antibodies were capable of blocking NK cell-mediated ADCC activity [155]. Another explanation for high plasma IgA levels seen in these higher risk individuals may simply be that they may have had greater levels of mucosal exposure and activation, resulting in a more robust systemic IgA response.

Aside from the immune correlates extracted from RV144, there are some other important features of this vaccine regimen that may have contributed towards the protection seen, and therefore should be considered. The first is that it was a heterologous prime-boost regimen, which may have reasonably contributed towards enhanced immune responses to a broader range of epitopes expressed from both the prime and the boost immunogens. In addition, the immunogens selected here were geographically relevant to the vaccinated population; CRF01_AE/Subtype B immunogens were used on a population primarily geographically affected by CRF01_AE and Subtype B HIV-1 infection [204]. For this reason, this vaccine can be considered to be a regionally-based vaccine rather than one that may have cross-protective efficacy in an area with a different predominant HIV-1 subtype. Our group has observed similar trends in the context of intrasubtype C superinfection, again strengthening the need for

wider evaluation of superinfection studies as a natural model in which to study HIV vaccination.

Extracting Vaccinology Lessons from HIV-1 Superinfection Studies

The fact that HIV-1 superinfection is a common occurrence indicates that the primary anti-HIV-1 immune response elicited in these individuals was insufficient to protect against HIV-1 reinfection and raises concerns about the feasibility of an HIV-1 vaccine. Although superinfection has historically been identified in high-risk individuals (CSW, MSM, IDU), we sought to identify superinfections within the context of relatively low-risk heterosexual couples, since most new primary infections occur in this cohort type worldwide. In sampling quarterly time points longitudinally with a combination of sequence-based approaches, we were able to confidently identify both the timing and the relative abundance of primary and superinfecting events. In addition, we were able to isolate viruses representative of the founder virus (or early founder-like variants) present at the time of seroconversions, as well as the superinfecting variant from the time that superinfection was first detected by phylogenetic confirmation.

We identified three intrasubtype C superinfected Zambian seroconverters (3/22 acute partners studied) that were superinfected during the first year of infection. All superinfections were genotypically characterized to be Subtype C, derived from a non-spousal (outside) partner, and resulted in rapid viral recombination between initial and superinfecting variants post-superinfection. These observations support the notion that even in a relatively low-risk cohort, sexual risk behavior of an individual or their social network [104] could play a role in superinfection, similar to what is seen in primary transmission. These data also underscore the necessity to understand dynamics of virus-host interactions both before and after superinfecting events, as superinfection and subsequent recombination have been shown to have serious clinical implications [55]. To

investigate further the question of why superinfections occur, we asked if individuals become superinfected because of a lack of cross-protection between the genetically disparate initial and superinfecting viruses, or if there was a specific immunological deficit in superinfected individuals that predisposes them to superinfection. The latter hypothesis was supported by the fact that all cases of superinfections occurred within the first year post-infection, despite longitudinal follow-up of up to 3-5 years, as well as the surprising finding that Env sequences in these individuals showed very little diversification prior to superinfection, consistent with low antibody pressure on the virus.

We compared the early immune responses (specifically evaluating anti-HIV humoral responses) in these three characterized superinfected cases to ten non-superinfected controls from the same cohort. In doing this, we sought to identify differences in primary immune responses to the first infection that could affect susceptibility to re-infection (or potentially primary infection in the extrapolated context of vaccination). Thus, these superinfected cases were treated as a natural model to study the immune correlates of protection that may be required in an effective and potentially cross-protective HIV-1 vaccine.

This body of work evaluates several arms of the antibody response normally seen during early HIV-1 infection, including autologous neutralization of founder viruses, development of Env-specific binding antibody responses and limited heterologous breadth against multiple subtype C viruses. We found that superinfected individuals exhibited low to undetectable levels of autologous neutralizing antibodies to their founder/early virus Env prior to superinfection, in contrast to the majority of non-superinfected individuals which showed early and potent neutralization of founder viruses similar to what has been shown previously in early Subtype C infection [127, 128]. We subsequently investigated heterologous neutralization breadth, as low levels of

cross-protecting antibodies could ostensibly affect superinfection susceptibility. Although we found no evidence of cross-neutralization of the superinfecting virus prior to superinfection, there was also no difference in the limited ability to cross-neutralize subtype C envelopes prior to superinfection between the superinfected and non-superinfected groups, indicating that ability to generate heterologous Nab breadth during early infection may not be a good predictor of susceptibility to superinfection during this early window.

In order to understand whether other potentially protective antibodies were compromised in superinfected individuals, we explored levels of Env-specific non-neutralizing antibodies. We found that superinfected individuals trended towards having lower gp120-specific (and a lack of V1V2-specific) binding antibody responses during early infection, suggesting a possible antibody deficit here as well. This was also the first independent study to our knowledge to corroborate a potential correlate of protection in V1V2 binding antibodies and a potential correlate of risk in high levels of anti-gp120 plasma IgA levels, first identified from the RV144 vaccine efficacy trials [93, 155]. However, without discrete mechanisms of action, further investigation is required to determine whether these correlates play a direct or indirect role in protection from superinfection.

Our data suggesting that a lack of protective antibodies during early infection can predispose individuals to superinfection is supported by another immunological study of 3 Subtype B MSM individuals, who were also superinfected during their first year of infection by heterologous Subtype B viruses [177]. Although this study is similar in that it shows low autologous neutralization of initial variants (isolated from the pre-superinfection time point) against contemporaneous pre-superinfection plasma, the study has some technical flaws. For example, it is unclear on what basis initial and superinfecting variants were chosen, heterologous breadth determinations were based

on neutralization of lab-adapted strains instead of clinically relevant primary isolates, and there was no evaluation of heterologous breadth to the superinfection variant prior to superinfection. Still, this study was the first to identify a potential neutralizing antibody deficit during early infection that may predispose individuals towards superinfection.

This is in contrast to the body of work evaluating superinfection in Kenyan female sex workers, which found no significant difference in heterologous neutralizing antibody breadth and potency prior to superinfection [171]. Heterologous breadth, rather than autologous neutralization of founder viruses or non-neutralizing antibodies, has been the focus of investigation in this Kenyan cohort. It is important to recognize some crucial differences that may partially account for this discrepant finding regarding levels of neutralizing antibodies prior to superinfection. These differences include the fact that multiple HIV-1 subtypes circulate in Kenya (e.g. A, C, D and various recombinants), the cohort examined is composed of sex workers (who are likely at greater risk of HIV-1 exposure in an endemic country), and superinfections in this cohort have been shown to occur both within the first year of infection and as late as 5+ years post-infection. As it is commonly recognized that the potential for neutralization breadth increases over time [132], the fact that there are no significant differences in heterologous breadth prior to superinfection (which occurs late into chronic infection) may reflect this temporal phenomena. Interestingly, lower heterologous breadth in the superinfected group as compared to case-matched controls was observed at one year post-infection, implying that there could also be an early humoral deficit in the individuals who became superinfected; however, the timing of superinfection may be affected by other factors (including stochastic factors like frequency of sex work, HIV partner status, etc.). In addition, a recent extended analysis of 146 women screened for superinfection from this same cohort concluded that the risk of superinfection is highest in early infection (within

the first two years) and may wane over time, in contrast to what the authors had previously described [216]. A mechanism for why this trend is seen has yet to be functionally investigated and defined, but this new data further supports our hypothesis that timing of superinfection may be related to early immune responses.

The role of non-neutralizing antibodies must not be overlooked either, as these binding antibodies could also mediate antiviral effector functions such as ADCC and sequestration of virus at the epithelial or mucosal barriers [151]. Although a few studies have shown that passive transfer of non-neutralizing antibodies could not protect against chimeric Simian-HIV (SHIV) virus infection in non-human primate models [201, 217], non-neutralizing antibody-mediated activity has recently been of great interest due to its implicated role in the protection seen from the RV144 vaccine previously discussed. Post-hoc analysis of the VAX004 vaccine trial, which showed no overall vaccine efficacy, also suggested that vaccinees that remained uninfected had higher levels of vaccine-induced antibody-dependent cell-mediated viral inhibition (ADCVI, a cumulative effect of ADCC, release of β-chemokines and phagocytosis) to a clinical isolate tested (HIV-$1_{92US657}$); however, these results were correlative rather than mechanistic [218]. By contrast, a recent publication again studying superinfected Kenyan sex workers did not find an association between ADCVI and superinfection outcome [219]. Although ADCC/ADCVI could be a mechanism of early viral control protecting against superinfecting viral outgrowth, it must be recognized that no human studies to date have directly shown ADCC to confer protection [220]. Therefore, its role in protection from superinfection is also less clear. Nevertheless, my recent studies have shown that pre-superinfection plasma samples from subsequently superinfected individuals show lower levels of ADCC activity than non-superinfected controls (Basu et al, manuscript in preparation). This deficit is despite normal levels of cytomegalovirus-specific IgG antibodies in these individuals during early infection (3 months after HIV-1

seroconversion), which may point to a compromised HIV-specific humoral response to primary HIV-1 infection in individuals that become superinfected, rather than a global immune defect.

It must be recognized that we were unable to obtain samples to evaluate cellular immune responses in these superinfected individuals. Despite this fact, based on the cumulative evidence discussed above, we can reasonably argue that antibodies are more likely to play a direct role in protection from HIV-1 acquisition. However, both arms of the immune system likely shape susceptibility to superinfection, control of superinfecting viral replication, subsequent immunopathology and clinical outcome, and it is likely that a deficit in either arm could affect the other in the context of superinfection. The humoral antibody response readouts measured here may also be reflective of other immunological problems in superinfected individuals, including those related to cellular immune responses.

For example, one reasonable hypothesis states that in certain individuals, early detrimental effects of HIV-1 infection (including compromised CD4+ T cell help) could preclude the ability to generate high-affinity antibodies through affinity maturation at the germinal centers of secondary lymphoid organs, which are also damaged by HIV-1 chronic immune activation. This cascade could subsequently delay the kinetics of a productive autologous neutralizing antibody response [38]. Such individuals may be at a higher risk of HIV-1 superinfection, especially during early infection, when protective antibodies (including neutralizing antibodies and binding antibodies) could be more critical. Perhaps over time, and despite its ultimate futility, HIV-specific humoral responses are able to re-establish themselves and potentially develop heterologous neutralization breadth to multiple HIV-1 variants. In the context of superinfections occurring late into chronic infection, this early humoral deficit may be overlooked. Another hypothesis is that the trend toward early superinfection may also be linked to

the availability of target CD4+ T cells, which are progressively depleted over the course of chronic HIV-1 infection [72]. Therefore, immune deficits in the cellular compartment may also play a significant role in predisposition to superinfection, but we currently lack availability of human samples to better address this contribution.

It is also possible that, like transmitted/founder viruses, evaluating genetic or biological signatures of founder and superinfecting variants may lend additional information about intrinsic characteristics of the viruses that may contribute towards their immunogenicity. Few studies to date have found that superinfecting variants can have a higher fitness than primary infecting variants [182]. However, in our studies, we have observed normal viral loads in superinfected individuals prior to superinfection, suggesting that the founder viruses evaluated do not likely have any overt defect in viral replication in vivo. Further evaluation of viral characteristics may help clarify potential viral factors affecting susceptibility to superinfection and control of superinfecting virus replication.

Aside from better understanding possible immune correlates of HIV-1 protection, here too we find some potential lessons from superinfection studies relevant to vaccination strategies. The first is that we find evidence of increased autologous neutralizing antibody titers following superinfection in the three superinfected individuals, indicating that although Nab responses were low pre-superinfection, this secondary infection may have still served as an immunological boost to the first infection, despite sequence disparities of 8-12% in Env. Smith et al. also observed a similar boost in autologous neutralization after superinfection in intrasubtype B superinfected MSM individuals [177]. Increased heterologous breadth after superinfection has also been observed in chronic Cameroonian superinfected individuals, and as well as Kenyan CSW [190, 191, 221]. These studies suggest that perhaps heterologous prime-boost regimens with immunological boosting during early

infection could help support high titers of protective antibodies in uninfected vaccinees; therefore, this regimen should continue to be pursued in vaccine regimens. A variation of this basic strategy will be tested in the RV306 clinical study (currently in phase I trials) where a 1-year boost will be added to the previous RV144 regimen in order to evaluate whether vaccine-induced immune responses are better sustained using this adapted treatment [200]. However, in this case the additional boost will be homologous rather than heterologous.

Finally, both our studies and those of RV144 suggest some potential for intrasubtype protection and may argue that designing regionally-based vaccines with immunogens relevant to subtypes specifically affecting certain epidemics may be efficacious in settings of relatively limited HIV-1 subtype diversity. However, in the setting of regions affected by multiple subtypes of HIV-1, a different strategy that addresses this challenging diversity may be required.

Summary

Despite small numbers, the correlation seen in our studies between autologous Nab titers and superinfection outcome, as well as the lack of additional superinfections after 1 year, suggests that poor early immune responses may predispose individuals towards superinfection. By contrast, high levels of antibodies could result in protection from superinfection, or they could represent an indirect surrogate of another protective immune factor. We have found that evaluating autologous neutralization (an *in vitro* representation of the naturally occurring Nab response to each patient's founder viruses) and binding antibodies (reflective of general HIV-specific humoral responses), rather than heterologous breadth (a rare phenomena to occur during early infection, if at all) may better predict an infected individual's HIV-specific humoral response capacity, and thus, their susceptibility to superinfection.

Our data suggests that in the context of a setting where a single subtype predominates, as is the case of Subtype C in Southern Africa, an early and potent antibody-mediated immune response to primary infection could potentially protect against intrasubtype superinfection. Importantly, these data lend hope to the feasibility of inducing an efficacious protective immune response via an HIV-1 vaccine, especially in the context of intrasubtype protection.

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