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Reduced frequency of HIV superinfection in a high-risk cohort in Zambia

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ABSTRACT

Rates of HIV-1 superinfection, re-infection with a genetically distinct virus despite HIV-1 specific immune responses, vary in different risk populations. We previously found the rates of superinfection were similar to primary HIV infection (PHI) in a Zambian heterosexual transmission cohort. Here, we conduct a similar analysis of 47 HIV-positive Zambians from an acute infection cohort with more frequent follow-up, all infected by non-spousal partners. We identified only one case of superinfection in the first two years, significantly fewer than in our previous study, which was likely due to increased counseling during acute infection and an overall population-wide decline in factors associated with HIV transmission. The predominant virus detected after superinfection was a recombinant of the transmitted founder (TF) and the superinfecting strain. The superinfected individual mounted a neutralizing antibody response to the primary TF virus, which remained TF-specific over time and even after superinfection, did not neutralize the superinfecting variant.

1. Introduction

HIV-1 superinfection is defined as a secondary HIV infection that occurs after the development of a primary HIV-specific immune response (Allen and Altfeld, 2003; Blackard et al., 2002; Blish et al., 2007; Chohan et al., 2010; Smith et al., 2005a; Smith et al., 2005b; van der Kuyl and Cornelissen, 2007). In 2002, the first case in humans was published describing an MSM with retroviral syndrome whose initial virus (subtype AE) was completely replaced by a subtype B variant leading to an increase in viral load and rapid loss of $CD4^+$ T cells (Jost et al., 2002). This observation, coupled with later findings of superinfection in larger cohorts (Gottlieb et al., 2004; Ramos et al., 2002), raised major concerns in the HIV vaccine field because it suggested that the immune response induced by natural infection may not be sufficient to protect against subsequent infections/challenges. However, in light of recent data that show superinfection risk decreases over the course of infection, it is likely that protection against reinfection is related to the maturity of the immune response (Basu et al., 2012; Chohan et al., 2010; Kraft et al., 2012; Ronen et al., 2017; Ronen et al., 2014; Smith et al., 2005a; Wagner et al., 2017). The identification of the immunologic and virologic factors that predispose individuals to superinfection may help elucidate some of the correlates of protection and further inform vaccine strategy and design in the future.

Since the discovery of HIV in the early 1980s, the humoral response to infection has been a major focus of research. The development of a global HIV-1 vaccine will likely require the induction of a broadly neutralizing antibody response (bnAb) to protect against infection with diverse viral variants; however, to date, generating these responses have been a great challenge. Interestingly, exposing the immune system to increased viral diversity early in HIV-1 infection may increase the likelihood of eliciting broad nAb responses (Bhiman et al., 2015; Cortez et al., 2012; Cortez et al., 2015; Smith and Derdeyn, 2015; Williams et al., 2018).

Despite evidence that nAb can protect against simian-human immunodeficiency virus (SHIV) infection in NHP models (Parren et al., 2001; Pauthner et al., 2019), it is not clear from human trials whether nAb responses alone are associated with risk reduction (Tomaras and Haynes, 2014). The only vaccine trial that has proved partially efficacious, RV144, demonstrated that non-nAb may play a significant role in HIV-1 transmission (Haynes et al., 2012). These data emphasize the need for investigating the full scope of the immune response to determine how each component contributes to blocking HIV-1 transmission.

In a previous report, we determined that the rate of superinfection

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was similar to PHI in heterosexual couples in Zambia (13.6% vs 7.8% respectively, per year) (Basu et al., 2012; Kraft et al., 2012). We detected 3 cases of superinfection from a small cohort of 22 newly infected individuals; in each case, superinfection was detected within the first year of infection. Each of these individuals mounted delayed, as well as lower neutralizing and non-neutralizing antibody responses prior to superinfection compared to matched controls, similar to more recent reports suggesting that early, robust immune responses may be important in preventing re-infection (Basu et al., 2012; Chohan et al., 2010; Smith et al., 2005a; Wagner et al., 2017). However, a more comprehensive study recently found no significant associations between humoral immune responses and superinfection (Ronen et al., 2017).

In the current study, the 47 subjects under investigation were all from a cohort initiated in 2005 (the IAVI Protocol C cohort) and the rate of superinfection was greatly reduced (2.1%) compared to our previous study. Superinfection did occur within the first year of infection; however, in this one case the nAb response was earlier and more robust than in the previously identified superinfected individuals. We demonstrate that this nAb response was specific to the TF virus and even after superinfection, the superinfecting variant was not neutralized by autologous plasma. Taken together, these data are consistent with other studies that show early nAb responses may not always correlate with protection against HIV superinfection (Blish et al., 2007).

2. Materials and methods

2.1. Ethics

The study protocols for human subjects were approved by the Office for Human Research Protections registered by the Institutional Review Boards at Emory University and the University of Zambia Research Ethics Committee. Signed written informed consent in local language was obtained from all participating couples.

2.2. Study participants

Although most infections in sub-Saharan Africa are acquired in marriage, approximately, 20% of infections are acquired from non-spousal partners, leading to an epidemiologically unlinked transmission pair with genetically distinct viruses (Basu et al., 2012; Kraft et al., 2012). Since sex outside the partnership is a major risk factor for superinfection, we again focused our initial screening efforts on these unlinked pairs (Basu et al., 2012; Kraft et al., 2012).

47 epidemiologically unlinked transmission pairs from Lusaka, Ndola, and Kitwe (Zambia), were enrolled into a prospective acute infection cohort (IAVI Protocol C) launched in 2005; all participants were HIV-negative at the time of enrollment. Cohort was gender-balanced (22M, 25F) and the median age was 33 years-old. The median days post-estimated date of infection (EDI) was 44 days and the median viral load at this time point was ~50,000 copies/mL. All but four individuals were infected with a subtype C viruses; those remaining were infected with recombinant viruses based on sequencing of the Pol and gp41 encoding regions (Supplemental Table 1).

Upon seroconversion of the negative partner, epidemiologic linkage of the virus in the newly infected partner to that of their spouse was determined by phylogenetic analyses of HIV-1 *env* (gp41) (Trask et al., 2002). According to the study protocol, couples returned to clinic for 7 follow-up visits within the first year of infection (more frequent than the previous heterosexual transmission (HT) cohort (Basu et al., 2012)). Each visit included: joint risk reduction counseling (couples' voluntary counseling and testing, CVCT), STI screening, condom provision, and the collection of clinical and behavioral data, including self-reported data on sexual exposure with and without condoms. Consistent with individuals receiving CVCT, most of the reported sexual acts with their co-habiting partner in the first year of infection were protected (with condom) as opposed to unprotected (without condom) (Supplemental Table 1). A total of 48.9% of individuals had signs or symptoms of genital inflammation or genital ulcerative disease and only 17% had at least one positive Trichomonas test during the first year of infection. While 11% were RPR positive at baseline, 15% had incident syphilis at least one time in the first year of infection. Although reporting of non-spousal partners was limited for a majority of the volunteers, two individuals, one which was the superinfection case, reported extensive sexual activity with outside partners (Allen et al., 2003).

Non-superinfected controls were matched to the superinfected individual based on the following criteria: subtype of infection (all subtype C), viral load at time of seroconversion, estimated time to infection, sample dates available, and when possible, cumulative risk scores (Supplemental Table 1).

2.3. Viral RNA extraction and PCR amplification

As previously described, viral RNA was extracted from longitudinal plasma samples and used to generate cDNA with the SuperScript III One-Step RT-PCR system with Platinum® Taq DNA Polymerase (ThermoFisher Scientific; Waltham, MA) (Basu et al., 2012; Kraft et al., 2012). Bulk PCR was performed to generate amplicons for gp41 and p17 using primers listed in Supplemental Methods.

For samples that failed one-step RT-PCR amplification, we decoupled cDNA synthesis from the first round of PCR by first generating cDNA with the SuperScript III RT System (ThermoFisher; Waltham, MA), then using cDNA in a subsequent PCR using Phusion High-Fidelity DNA Polymerase (New England BioLabs; Ipswich, MA). Both PCR were followed by a nested second round of PCR (primers listed in Supplemental Methods).

2.4. DNA sequencing and phylogenetic analysis

Positive amplicons were pooled and purified using the Wizard SV Gel and PCR Clean Up System according to the manufacturer's instructions (Promega; Madison, WI). Purified DNA was submitted for Sanger sequencing on ABI 3730xl DNA Analyzer (GenScript; Piscataway, NJ).

Population sequences were edited and assembled using Sequencher 5.2 (Gene Codes Corporation, Ann Arbor, MI) and complete sequences were aligned and analyzed using Geneious R6 (Biomatters Ltd, Auckland, New Zealand). Alignments and Maximum Likelihood phylogenetic trees were generated using FastTree version 2.1.5, which uses the Jukes-Cantor model of nucleotide evolution and the "CAT" approximation to account for varying rates of evolution across sites.

Longitudinal sequences where Pairwise Distance (PWD) exceeded 5% were classified as "suspected cases of superinfection".

2.5. Superinfection detection

As previously described, longitudinal full-length *env* single genome amplification (SGA) was performed for suspected cases of superinfection (Kraft et al., 2012). Amplicons were analyzed using the Highlighter tool from the LANL HIV Sequence Database (hiv.lanl.gov) to monitor *env* evolution over time. Highlighter plots were generated by comparing longitudinal *env* sequences to the TF *env* sequence (master sequence); each colored hatch-mark represents a single nucleotide change from the TF *env* sequence (red = T, green = A, orange = G, light blue = C, dark blue = IUPAC/degenerate bases).

2.6. TOPO cloning and env plasmid generation

Previously identified full-length *env* SGA amplicons were re-amplified with TOPO-TA cloning primers; patient-specific primers were designed to generate amplicons for the superinfecting variant (See Supplemental Methods). 10 SGA amplicons were generated from the

seroconversion time point, and the sequences were aligned to establish a consensus sequence. The amplicon most similar to the consensus was purified with the Wizard SV Gel and PCR Clean Up system as previously described. Additionally, 8 full-length *env* SGA amplicons were also generated for the superinfection time point (greatest difference in PWD as compared to the seroconversion consensus sequence). Full-length *envs* were directionally subcloned into the pcDNA3.1 backbone using the pcDNA3.1 Directional TOPO Expression Kit (ThermoFisher Scientific; Waltham, MA) according to manufacturer's protocol and transformed as previously described (Basu et al., 2012; Kraft et al., 2012). Transformants were screened for the insert and miniprepped using the Pureyield Plasmid Miniprep System (ThermoFisher Scientific; Waltham, MA).

2.7. Function screening of full-length env clones and pseudovirus generation

Full-length Env clones were screened for infectivity and used to generate patient-specific Env pseudoviruses (Basu et al., 2012; Kraft et al., 2012). All functional clones were re-sequenced to confirm their identity.

2.8. Neutralization assays

Neutralization assays were performed in accordance with previously published studies (Basu et al., 2012; Kraft et al., 2012). Luciferase measurements were conducted using the Cytation3 plate reader and quantified using the Gen5 (v2.07 and v3.02) software (BioTek; Winooski, VT). Infectivity curves were generated using Prism (v6.0d and v7.0c) software (GraphPad; La Jolla, CA). The 50% inhibitory concentrations (IC50), which represents the plasma dilution resulting in 50% viral infectivity, were determined using the growth function in Microsoft Excel. Each Env-plasma combination was performed in duplicate for each independent experiment. Experiments were repeated at least twice.

2.9. HIV-1 IgG-gp120 binding ELISAs

gp120 ELISAs were performed as previously described (Basu et al., 2012) and were read at 450 nm absorbance using the Cytation3 plate reader and analyzed using Gen5 v2.07 software. Background was defined as the average absorbance from wells blocked with 1X B3T buffer but not incubated with plasma. This value was subtracted from each experimental well. Additionally, average absorbance from wells blocked with 1X B3T and incubated with normal human plasma (NHP; 1:100) was multiplied by 5; this value was considered the lower limit of detection.

2.10. Pac-Bio sequencing and data analysis

2.10.1. Longitudinal full-length env PCR amplification for superinfected individual (ZM1599F)

Full-length *env* SGA amplicons from seroconversion (SC) through 12mo post-infection were re-amplified using barcoded 2nd round nested primers with a distinct barcode for each time point (See Supplemental Methods).

2.10.2. Library preparation and Pac-Bio sequencing of FL env SGA or Near-full length genomes (NFLG) for superinfected individual (ZM1599F)

Purified NFLG SGA from the SC and 12mo time points or barcoded FL *env* SGA from longitudinal time points were included in separate SMRTbell libraries (see below) per manufacturer's protocol (Pacific Biosciences Inc., CA) in equal concentrations for a final concentration of 10 μg (Dilernia et al., 2015).

Sequence derivation from the raw PacBio data was performed using the MDPseq software pipeline (Dilernia et al., 2015). To analyze the output sequences, amplicons were initially organized by barcode and aligned using Geneious software; only amplicons with greater than 40 reads were included in the final analysis. Consensus sequences were generated for each time point and compared to the TF virus sequences using Highlighter.

2.11. Statistical analyses

2.11.1. 2x2 contingency analysis

To determine whether superinfection incidence was statistically different between the two heterosexual transmission cohorts: (HT; pre-2005/early) versus Protocol C (post-2005/late) cohorts, we first performed a contingency analysis. The p-value was determined using a two-tailed Fisher's exact test.

2.11.2. Behavioral data analysis

For all variables in Supplemental Table 1, we performed Mann-Whitney unpaired U-tests in GraphPad Prism 7.0c to compare the medians between the early (HT) and late (Protocol C) cohorts.

3. Results

3.1. Detection of superinfection

We previously identified 3 cases of superinfection from a small subset of serodiscordant couples from the Zambia-Emory HIV Research Program (ZEHRP) and determined that these individuals were superinfected with subtype C viruses from non-spousal partners within the first year (3–10 months post-seroconversion) of infection.

In order to determine whether the rate of superinfection in our initial study of 22 individuals was representative of a larger population, we screened an additional 47 volunteers for evidence of superinfection by amplifying and sequencing gp41 from longitudinal samples through two-years post-seroconversion (Supplemental Table 1). Phylogenetic analyses revealed that only 1 of the 47 newly analyzed individuals had evidence of superinfection (frequency = ~2.1% [95% CI 0.1–11.1]) (Fig. 1A; Supplemental Fig. 1).

In the newly identified superinfected individual, ZM1599F, changes in gp41 were minimal through 9-months post-seroconversion (Fig. 1B). Early variants never differed more than 0.5% by pairwise distance (PWD) from the TF, consistent with the relatively low rate of diversification for this region (Zanini et al., 2015). However, the viral variant detected at 12 months differed 11.4% from the founder, demonstrating the introduction of a distinct virus between 9 and 12 months. Viruses amplified at 24 months were 98.9% identical to the 12-month virus, ruling out the possibility of a transient superinfection.

To confirm the presence of superinfection and thus rule out possible contamination, we performed SGA and single molecule, long-read (SMLR) sequencing of full-length *env* from longitudinal samples between the time of transmission and superinfection (Fig. 1C). Consistent with gp41, there was minimal envelope diversification in the first 9 months of infection resulting in a maximum PWD across full-length *env* of less than 1%, the result of normal evolutionary pressure. In contrast, the virus detected at 12 months contained over 350 nucleotide changes with a PWD of 13.5% likely due to the introduction of a distinct viral variant. These observations of limited gp41 and *env* diversification prior to superinfection are consistent with viral population dynamics observed in the previously identified SI cases in the HT cohort (Basu et al., 2012; Kraft et al., 2012).

3.2. Characterization of superinfection

As superinfection can result in *in vivo* recombination (Chaillon et al., 2013; Fang et al., 2004; Koning et al., 2013), we also amplified and sequenced an approximately 399 bp fragment of gag (p17) at each time point through the first 2 years of infection. The p17 sequence remained TF-like, differing by less than 0.1% through 12-months post-

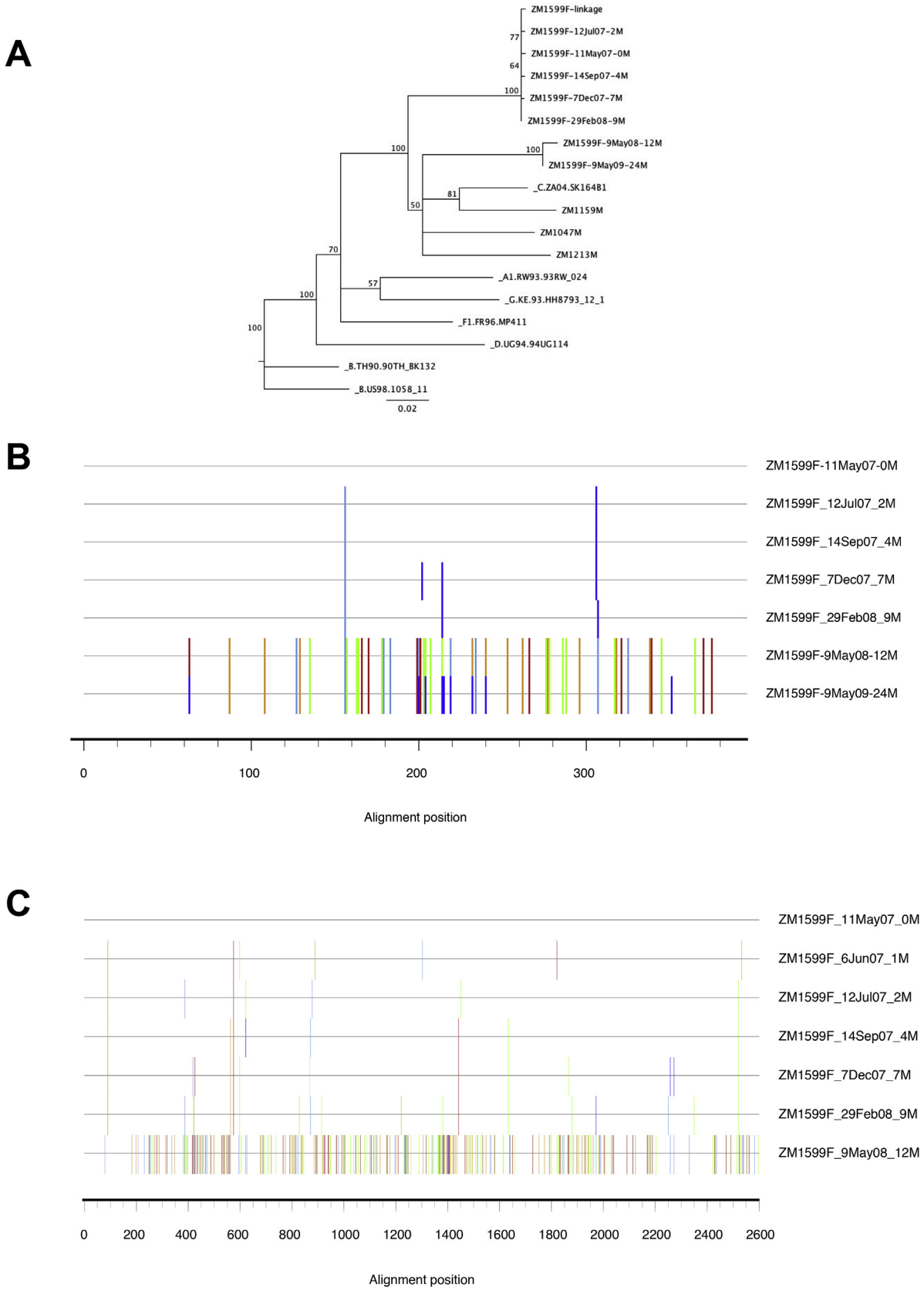

Fig. 1. Minimal changes in early Env sequences prior to superinfection.
Longitudinal gp41 population sequences were analyzed using a neighbor-joining phylogenetic approach for the one identified case of superinfection, ZM1599F (A). gp41 population sequences (B) and full-length envelope single genome sequences (SGA) (C) for longitudinal samples (FL env amplicons were barcoded by time-point and sequenced using the NGS platform, PacBio) were examined using an HIV Los Alamos National Laboratory (LANL) tool, Highlighter, to visualize viral evolution in envelope early in infection. Sequences were compared to the inferred TF virus (0M); mismatches are represented by colored hatch-marks (green = A, light blue = C, orange = G, red = T, dark blue = IUPAC)

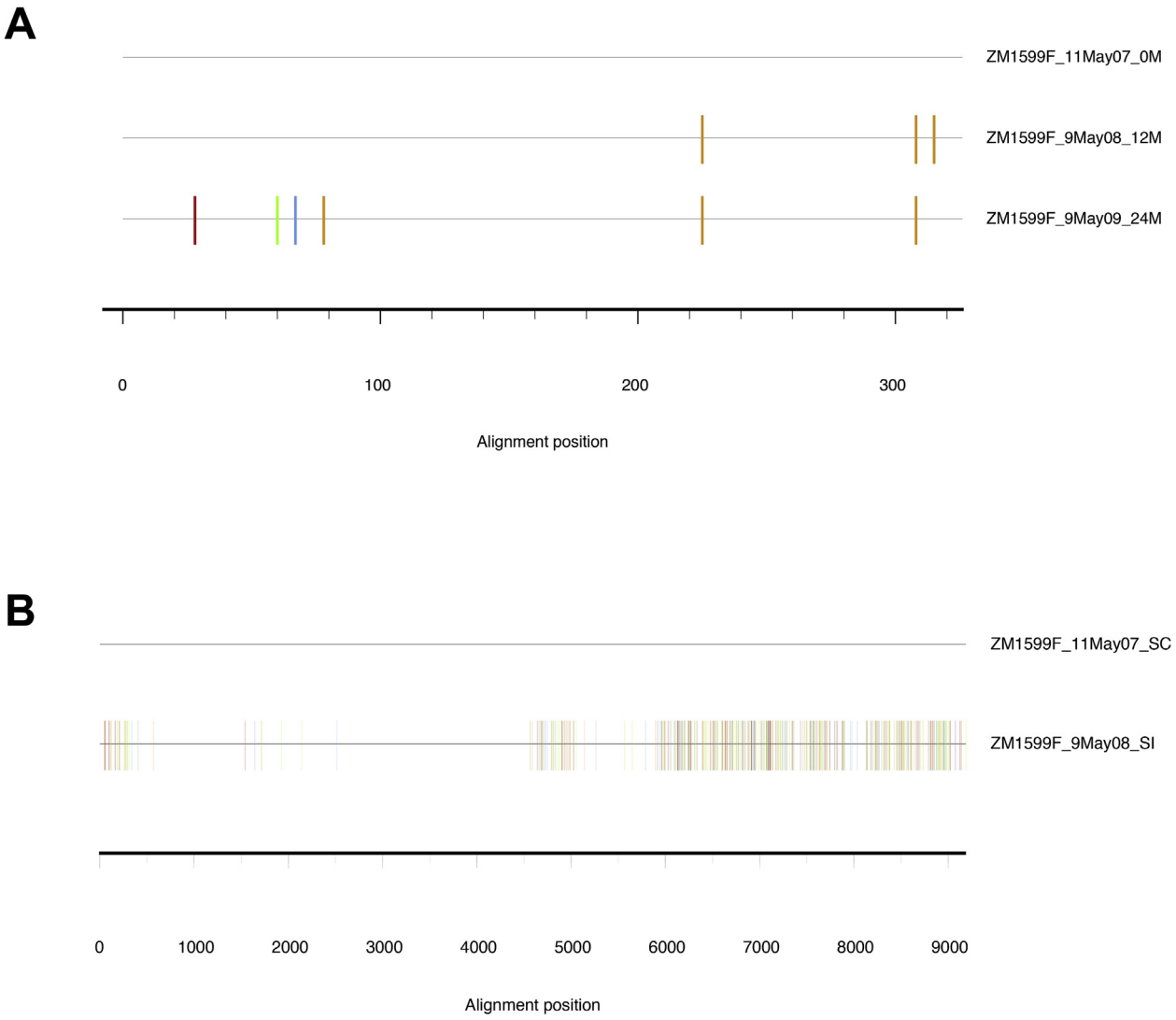

Fig. 2. Recombination detected after superinfection.
Gag (p17) population sequences for longitudinal samples were examined using the Highlighter tool to visualize viral evolution in the 5' half of the HIV-1 genome (A). Near FL HIV-1 SGA sequences were also amplified for both the seroconversion time point (0M; SC) and the 1st time point post-superinfection (12M; SI) and sequenced using PacBio (B). Sequences were examined using the Highlighter tool to detect changes along the entire length of the HIV-1 genome. Sequences were compared to the inferred TF virus (0M). Mismatches are represented by colored hatch-marks (green = A, light blue = C, orange = G, red = T, dark blue = IUPAC).

seroconversion (Fig. 2A; Supplemental Fig. 2), suggesting that the virus detected at 12 months was likely a recombinant containing a conserved *gag* and highly diversified/distinct *env*. To test this hypothesis, we sequenced near full-length genomes from seroconversion (0 months; SC) and superinfection (12 months; SI) using a SMLR approach (Fig. 2B). Similar to our previously identified superinfection cases, both variants were subtype C suggesting intrasubtype superinfection. Most of the genomic changes observed occurred in the 3′-end of the genome, although there were some changes in the 5′- untranslated region of the genome. The *gag-pol* region and some of the accessory genes at the 3′-end, *vpr, vpu, tat,* and *rev,* were highly conserved between the TF and the virus observed at 12 months (8 nucleotide difference in *gag-pol*). However, *vif*, *env*, and *nef* in the 12-month virus (SI) were genetically distinct from those in the TF (SC), suggesting that these genes were derived from a superinfecting variant (Fig. 2B). Due to the limited frequency of sampling, we were unable to identify the virus that initiated superinfection, but these data clearly suggest that recombination occurred sometime after superinfection (between 9 and 12 months post-infection) to yield the dominant variant detected at 12 months. Further, although the gp41 population sequencing results suggest that the superinfecting variant effectively replaced the TF virus, our ability to amplify full-length envelope from the TF at 12 months indicates that both TF and the recombinant virus were circulating after superinfection.

3.3. Characterization of humoral immune responses in superinfection

In order to determine if a reduced antibody response might have predisposed ZM1599F to superinfection, we measured nAb responses in longitudinal samples prior to superinfection. Similar to previous work, the kinetics and magnitude of the autologous nAb response were variable amongst the singly-infected controls (Fig. 3A, black lines), with neutralization titers exceeding background levels (IC50 > 100) by six-months post-infection for 11 out of 13 controls (Li et al., 2006). The neutralization titers for the remaining two controls did not exceed background in the first year following infection (Fig. 3A, gray lines).

In contrast to delayed nAb responses observed in our previous superinfection cases (Basu et al., 2012), ZM1599F mounted an early autologous nAb response to the TF, similar to the majority of the non-superinfected controls, and the nAb titer was close to the median IC50

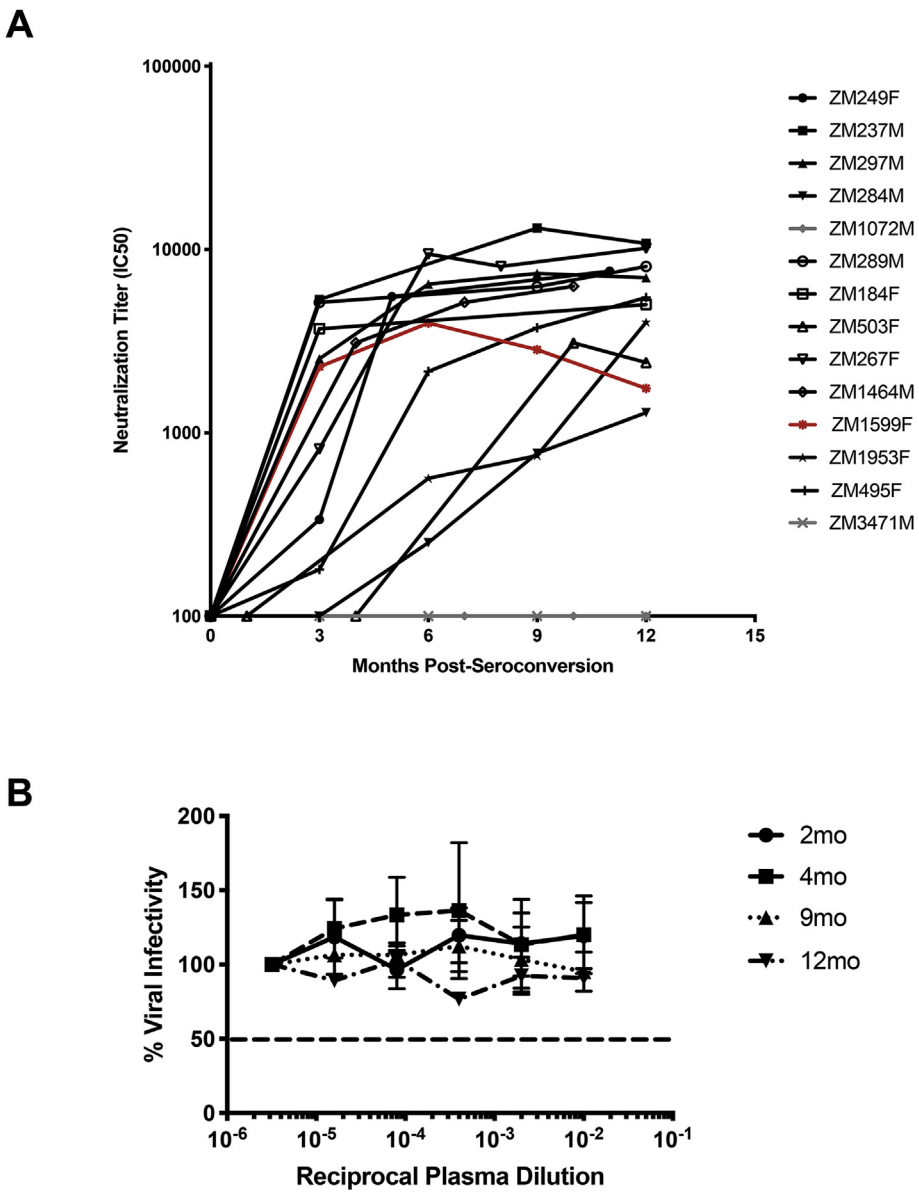

Fig. 3. Wide variability in the timing and potency of the autologous neutralizing antibody response to TF viruses.
TF Env pseudoviruses were tested for neutralization by longitudinal autologous plasma samples from 13 non-superinfected/singly-infected controls (black and gray lines; gray lines represent individuals where IC50 titers never exceeded the minimum threshold) and one case of superinfection, ZM1599F (red line). Plasma neutralizing antibody IC50 titers are displayed for each autologous plasma-TF pseudovirus pair in (A). The superinfecting Env pseudoviruses from ZM1599F was tested for neutralization by longitudinal plasma samples to evaluate autologous neutralizing antibody responses in the first year of infection (B). Dotted horizontal black line denotes where 50% viral infectivity is achieved. Each neutralization assay was conducted in duplicate and graphs represent the median of two independent experiments.

at 3 months post-PHI. Although, the nAb titers increased through six months, they fell below the median nAb titer at 9, and 12-months post-infection.

In addition, while the TF could be neutralized prior to superinfection, the superinfecting variant was not neutralized by autologous plasma in the first year (Fig. 3B). These data support previous findings that show, though early nAb responses are potent, they tend to be restricted to TF viruses in the first 12 months post-infection (Gray et al., 2007; Moore et al., 2009; Wagner et al., 2017); and indeed, ZM1599F pre-superinfection plasma showed limited neutralization breadth against subtype C viruses from a panel of Tier 1b and Tier 2 viruses (Fig. 4A). Moreover, plasma from ZM1599F was unable to neutralize the superinfecting variant even through two-years post-infection (Fig. 4B). In contrast, antibodies with the capacity to neutralize the TF at dilutions > 1:1000 were present through 24-months (Fig. 4C). The superinfecting virus could, however, be neutralized as efficiently as TF viruses from three non-superinfected controls (Fig. 4D) by a pool of plasma from 20 subtype-C infected individuals from Zambia. These data showed that this virus was not uniquely resistant to neutralization, rather that ZM1599F was simply unable to produce neutralizing antibodies to the Env of the superinfecting virus.

Since the humoral response is not limited to neutralizing antibodies, it was important to consider how other types of antibodies might play a role in superinfection. To determine if these antibodies might be lower in superinfected individuals, we therefore measured plasma IgG binding to a heterologous Env in pre-superinfection samples; we also tested plasma from non-superinfected controls at similar time points (Fig. 5). We observed maximal gp120-IgG binding with autologous plasma (ZM205F), and minimal binding from the plasma of an HIV-1 exposed seronegative (ESN) individual. While early plasma (2mo) from ZM1599F, displayed minimal gp120-binding, plasma from the pre-superinfection time-point showed binding comparable to the 10 non-superinfected control individuals.

3.4. Comparison of superinfection incidence in HT and Protocol C cohorts

The finding of only a single case of superinfection out of 47 Protocol C participants was unexpected, given our previous study of superinfection, where we observed superinfection in 3/22 participants in the first year following PHI. A further breakdown of the participants in our previous study showed that 8 of the 22 were also enrolled in Protocol C, and that all three superinfections occurred in participants of the earlier

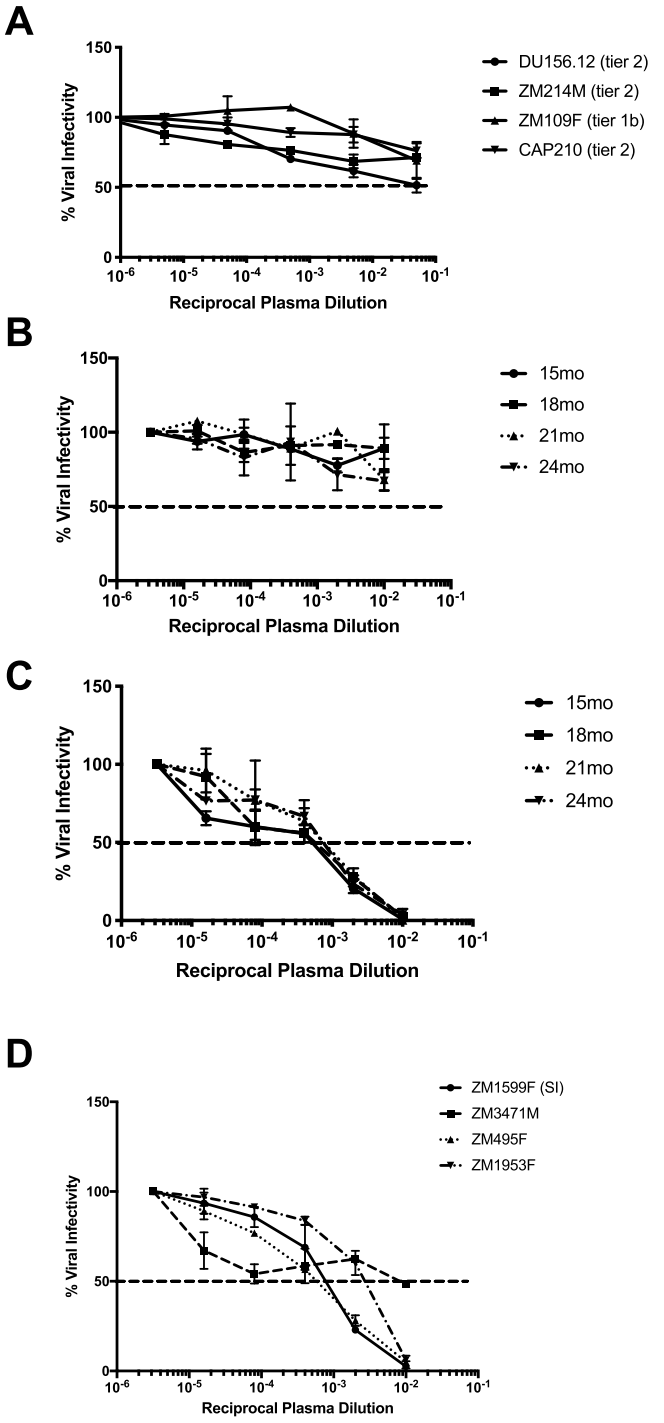

Fig. 4. Autologous neutralizing antibody response remains strain-specific over the first 2 years of infection.
Representative graph of plasma from the time point prior to superinfection was tested for neutralization against 4 pseudoviruses randomly selected from a subtype C panel to include viruses with tier 1 and tier 2 sensitivities (A). Superinfecting (B) and TF (C) Env pseudoviruses were tested for neutralization by longitudinal plasma samples to evaluate autologous neutralizing antibody responses in the second year of infection (12–24M). Env pseudoviruses from ZM1599F (superinfecting variant) and 3 non-superinfected controls were used to assess neutralization against a plasma pool from 20 subtype C HIV-1 infected Zambians (D). Dotted horizontal black line denotes where 50% viral infectivity is achieved. Each neutralization assay was conducted in duplicate and graphs represent the median of two independent experiments.

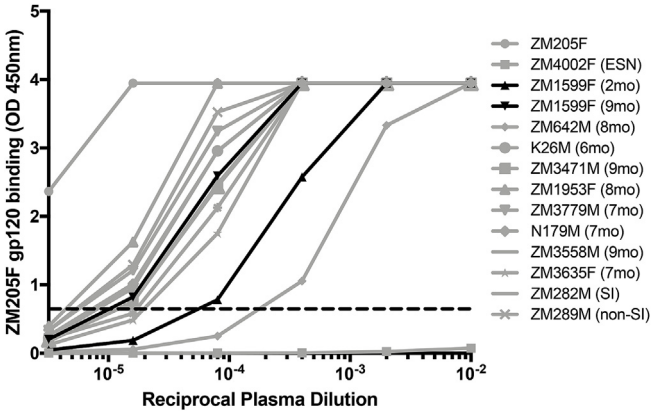

Fig. 5. Levels of plasma IgG binding antibody to heterologous gp120 antibody were not statistically different from non-superinfected controls.
Serial plasma dilutions were used in a gp120 binding ELISA. The source of purified gp120 protein was a Zambian subtype C seroconvertor, ZM205F; autologous plasma from this individual was used as a positive control for the presence of gp120-specific binding antibodies. Gray lines represent non-superinfected controls. Dotted horizontal black line represents IgG binding threshold above background. Each ELISA was conducted in duplicate and graphs represent the median of two independent experiments.

HT cohort. Thus 75% percent (3/4) of the superinfection cases identified to-date in our Zambian studies come from the earlier cohort and were all pre-2005. In contrast, all individuals in the current study were from the Protocol C cohort and samples were obtained from 2005 to 2010. We therefore performed a 2x2 contingency analysis using Fisher's exact test to compare the number of superinfection cases identified in the HT (3/14) versus Protocol C (1/55) cohorts and showed a statistically different superinfection frequency ($p = 0.02$) between them. Although based on a single case of superinfection, it should be noted that even if we had observed an additional case (i.e. 2/55), the difference in superinfection frequencies would have remained statistically significant ($p = 0.05$).

We next performed a meta-analysis to compare STI incidence (RPR and Trichomonas), genital ulceration/inflammation, outside partners, and sex with or without (+/-) condoms in the two cohorts. In addition, since Protocol C required more frequent visits in the first year of infection and these visits included risk reduction counseling, we also included this variable in our analysis (Supplemental Table 1). We did not observe significant differences in a majority of the variables; however, the number of study/CVCT visits in the first-year post-infection was statistically different ($p < 0.0001$).

4. Discussion

4.1. Frequency of superinfection

While most of what is known about superinfection comes from higher risk individuals from female sex worker (FSW), MSM, and IDU cohorts, we sought to define superinfection frequency in a discordant heterosexual couple cohort as this group contributes the largest number of new infections to the epidemic in Africa (Dunkle et al., 2008).

In this study, where individuals were initially infected with HIV from an outside partner, we identified only one case of superinfection during the first two years. Although consistent with more recent reports from a cohort with the largest number of superinfection cases recorded in the literature, it differed from our previous publication where we found no statistical difference between superinfection frequency and PHI incidence (Kraft et al., 2012). This difference between our two superinfection studies is likely attributable to study protocol, which for the current study included significantly more clinic visits in the first year of infection (5.7 versus 3.5 visits in the previous study), when

superinfection risk is highest. Since these visits include risk-reduction counseling and testing (couples' voluntary counseling and testing; CVCT), which has been shown to reduce HIV transmission by up to 70% (Dunkle et al., 2008), it is possible that these additional visits served to prevent re-infection until the individuals mounted their own HIV-specific immune responses. In addition, because secular trends associated with HIV transmission and acquisition were declining and ART usage was increasing, the likelihood of superinfection in this current study would be negatively impacted (Wall et al., 2017b). Together, these factors likely explain why superinfection frequency declined over time in our cohorts.

4.2. Recombination following superinfection

In ZM1599F we detected clear evidence for superinfection in *env* (gp41) and an absence in *gag* (p17). Amplifying and sequencing near full-length genome amplicons confirmed that the virus detected at 12 months post-PHI was a recombinant that contained a TF-like 5′-end and a genetically distinct 3′-end. Although, this is best explained by recombination following superinfection, we were unable to detect the original genomic sequence of the superinfecting virus due to limited sampling frequency. It is of interest that the recombinant retained sequences in the 5′ LTR-UTR region, the Vif coding region, most of the Env coding region, as well as the Nef coding region from the genetically distinct superinfecting virus. Given the lack of nAb responses to the superinfecting virus, the retention of the superinfecting virus Env would be expected to give both virus and infected cells a survival advantage. Presumably the 5′LTR-UTR and accessory genes like Vif and Nef of the superinfecting virus also contribute to an enhanced fitness for the superinfection recombinant. Together, these data support previous findings that point to superinfection as a common source of recombination; these new recombinant viruses are often more fit (Gordon et al., 2016) and capable of modulating host responses to prevent immune recognition (Courtney et al., 2017).

4.3. Humoral responses to superinfection

Based on previous work from our laboratory, we expected superinfection to result in delayed, lower nAb responses to the TF. However, in the current study, ZM1599F mounted an early autologous nAb response to the TF, similar to the majority of the non-superinfected controls post-PHI. This corroborates other findings which suggest that individuals that mount early humoral responses with nAb titers close to the median can still be superinfected (Blish et al., 2007; Blish et al., 2008; Ronen et al., 2017).

The nAb response in ZM1599F remained TF-specific with no significant neutralization of the superinfecting Env pseudovirus even one-year post-superinfection, even though the latter was sensitive to neutralization by pooled plasma. Although we have not investigated the breadth of antibody responses to a panel of other HIV-1 envelopes post-superinfection in ZM1599F, this inability to neutralize the superinfecting virus is quite different from the *de novo* responses seen post-superinfection in a South African subtype C cohort (Sheward et al., 2018). It also differs from the increased polyclonal response seen following inter-subtype superinfection (Cortez et al., 2012; Cortez et al., 2015; Powell et al., 2010).

Non-neutralizing antibodies (nnAb) can elicit Fc-mediated effector functions that can also play a role in protection from infection. Some studies, including our previous study in Zambia, suggest that reduced levels of Env-binding Ab are correlated with superinfection (Basu et al., 2012; Basu et al., 2014; Wagner et al., 2017; Wall et al., 2017a). However, others, including the current study, have not found significant differences in nnAb between singly-infected controls and superinfected cases prior to superinfection (Forthal et al., 2013; Ronen et al., 2017), though a recent paper highlights the importance of the Env antigens used to probe these responses in interpreting these results (Courtney et al., 2017). Together, these data suggest that though nnAb may protect against PHI in a vaccine setting, nnAb may not always correlate with protection against re-infection.

In conclusion, we observed a low incidence of superinfection in a cohort of acutely infected individuals who had demonstrated high risk behavior through HIV-1 infection by a non-spousal partner. It is likely that a more frequent counseling schedule, coupled with decreasing risk factors associated with HIV transmission in the cohort contributed to this low incidence. Although our previous studies suggested diminished humoral responses following PHI may have increased susceptibility to superinfection, the single case of superinfection reported here is consistent with more recent findings that suggest superinfection can occur despite early, robust, and broad nAb responses to founder virus (Ronen et al., 2017; Serwanga et al., 2018). Nevertheless, the initial nAb response in ZM1599F was and remained TF virus-specific despite the neutralization sensitivity of the superinfecting variant to a pool of plasma from subtype C infected individuals, suggesting a limited ability to target the superinfecting variant. Together, this study, along with other previously published results, suggests that though humoral immunity may play a significant role in preventing PHI, the timing, specificity, and potency of these early responses may not always be sufficient to prevent secondary HIV-1 infections.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.06.009.

References

Allen, S., Meinzen-Derr, J., Kautzman, M., Zulu, I., Trask, S., Fideli, U., Musonda, R., Kasolo, F., Gao, F., Haworth, A., 2003. Sexual behavior of HIV discordant couples after HIV counseling and testing. AIDS 17, 733–740.

Allen, T.M., Altfeld, M., 2003. HIV-1 superinfection. J. Allergy Clin. Immunol. 112, 829–835 quiz 836.

Basu, D., Kraft, C.S., Murphy, M.K., Campbell, P.J., Yu, T., Hraber, P.T., Irene, C., Pinter, A., Chomba, E., Mulenga, J., Kilembe, W., Allen, S.A., Derdeyn, C.A., Hunter, E., 2012. HIV-1 subtype C superinfected individuals mount low autologous neutralizing antibody responses prior to intrasubtype superinfection. Retrovirology 9, 76.

Basu, D., Xiao, P., Ende, Z., Bere, A., Britt, W.J., Mulenga, J., Kilembe, W., Allen, S.A., Derdeyn, C.A., Hunter, E., 2014. Low antibody-dependent cellular cytotoxicity

responses in Zambians prior to HIV-1 intrasubtype C superinfection. Virology 462–463, 295–298.
Bhiman, J.N., Anthony, C., Doria-Rose, N.A., Karimanzira, O., Schramm, C.A., Khoza, T., Kitchin, D., Botha, G., Gorman, J., Garrett, N.J., Abdool Karim, S.S., Shapiro, L., Williamson, C., Kwong, P.D., Mascola, J.R., Morris, L., Moore, P.L., 2015. Viral variants that initiate and drive maturation of V1V2-directed HIV-1 broadly neutralizing antibodies. Nat. Med. 21, 1332–1336.
Blackard, J.T., Cohen, D.E., Mayer, K.H., 2002. Human immunodeficiency virus superinfection and recombination: current state of knowledge and potential clinical consequences. Clin. Infect. Dis. 34, 1108–1114.
Blish, C.A., Blay, W.M., Haigwood, N.L., Overbaugh, J., 2007. Transmission of HIV-1 in the face of neutralizing antibodies. Curr. HIV Res. 5, 578–587.
Blish, C.A., Dogan, O.C., Derby, N.R., Nguyen, M.A., Chohan, B., Richardson, B.A., Overbaugh, J., 2008. Human immunodeficiency virus type 1 superinfection occurs despite relatively robust neutralizing antibody responses. J. Virol. 82, 12094–12103.
Chaillon, A., Wagner, G.A., Hepler, N.L., Little, S.J., Kosakovsky Pond, S.L., Caballero, G., Pacold, M.E., Phung, P., Wrin, T., Richman, D.D., Wertheim, J.O., Smith, D.M., 2013. Dynamics of viral evolution and neutralizing antibody response after HIV-1 superinfection. J. Virol. 87, 12737–12744.
Chohan, B.H., Piantadosi, A., Overbaugh, J., 2010. HIV-1 superinfection and its implications for vaccine design. Curr. HIV Res. 8, 596–601.
Cortez, V., Odem-Davis, K., McClelland, R.S., Jaoko, W., Overbaugh, J., 2012. HIV-1 superinfection in women broadens and strengthens the neutralizing antibody response. PLoS Pathog. 8, e1002611.
Cortez, V., Wang, B., Dingens, A., Chen, M.M., Ronen, K., Georgiev, I.S., McClelland, R.S., Overbaugh, J., 2015. The broad neutralizing antibody responses after HIV-1 superinfection are not dominated by antibodies directed to epitopes common in single infection. PLoS Pathog. 11, e1004973.
Courtney, C.R., Mayr, L., Nanfack, A.J., Banin, A.N., Tuen, M., Pan, R., Jiang, X., Kong, X.P., Kirkpatrick, A.R., Bruno, D., Martens, C.A., Sykora, L., Porcella, S.F., Redd, A.D., Quinn, T.C., Nyambi, P.N., Durr, R., 2017. Contrasting antibody responses to intrasubtype superinfection with CRF02_AG. PLoS One 12, e0173705.
Dilernia, D.A., Chien, J.T., Monaco, D.C., Brown, M.P., Ende, Z., Deymier, M.J., Yue, L., Paxinos, E.E., Allen, S., Tirado-Ramos, A., Hunter, E., 2015. Multiplexed highly-accurate DNA sequencing of closely-related HIV-1 variants using continuous long reads from single molecule, real-time sequencing. Nucleic Acids Res. 43, e129.
Dunkle, K.L., Stephenson, R., Karita, E., Chomba, E., Kayitenkore, K., Vwalika, C., Greenberg, L., Allen, S., 2008. New heterosexually transmitted HIV infections in married or cohabiting couples in urban Zambia and Rwanda: an analysis of survey and clinical data. Lancet 371, 2183–2191.
Fang, G., Weiser, B., Kuiken, C., Philpott, S.M., Rowland-Jones, S., Plummer, F., Kimani, J., Shi, B., Kaul, R., Bwayo, J., Anzala, O., Burger, H., 2004. Recombination following superinfection by HIV-1. AIDS 18, 153–159.
Forthal, D.N., Landucci, G., Chohan, B., Richardson, B.A., McClelland, R.S., Jaoko, W., Blish, C., Overbaugh, J., 2013. Antibody-dependent cell-mediated virus inhibition antibody activity does not correlate with risk of HIV-1 superinfection. J. Acquir. Immune Defic. Syndr. 63, 31–33.
Gordon, K., Omar, S., Nofemela, A., Bandawe, G., Williamson, C., Woodman, Z., 2016. Short communication: a recombinant variant with increased envelope entry efficiency emerged during early infection of an HIV-1 subtype C dual infected rapid progressor. AIDS Res Hum Retroviruses 32, 303–310.
Gottlieb, G.S., Nickle, D.C., Jensen, M.A., Wong, K.G., Grobler, J., Li, F., Liu, S.L., Rademeyer, C., Learn, G.H., Karim, S.S., Williamson, C., Corey, L., Margolick, J.B., Mullins, J.I., 2004. Dual HIV-1 infection associated with rapid disease progression. Lancet 363, 619–622.
Gray, E.S., Moore, P.L., Choge, I.A., Decker, J.M., Bibollet-Ruche, F., Li, H., Leseka, N., Treurnicht, F., Mlisana, K., Shaw, G.M., Karim, S.S., Williamson, C., Morris, L., Team, C.S., 2007. Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. J. Virol. 81, 6187–6196.
Haynes, B.F., Gilbert, P.B., McElrath, M.J., Zolla-Pazner, S., Tomaras, G.D., Alam, S.M., Evans, D.T., Montefiori, D.C., Karnasuta, C., Sutthent, R., Liao, H.X., DeVico, A.L., Lewis, G.K., Williams, C., Pinter, A., Fong, Y., Janes, H., DeCamp, A., Huang, Y., Rao, M., Billings, E., Karasavvas, N., Robb, M.L., Ngauy, V., de Souza, M.S., Paris, R., Ferrari, G., Bailer, R.T., Soderberg, K.A., Andrews, C., Berman, P.W., Frahm, N., De Rosa, S.C., Alpert, M.D., Yates, N.L., Shen, X., Koup, R.A., Pitisuttithum, P., Kaewkungwal, J., Nitayaphan, S., Rerks-Ngarm, S., Michael, N.L., Kim, J.H., 2012. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N. Engl. J. Med. 366, 1275–1286.
Jost, S., Bernard, M.C., Kaiser, L., Yerly, S., Hirschel, B., Samri, A., Autran, B., Goh, L.E., Perrin, L., 2002. A patient with HIV-1 superinfection. N. Engl. J. Med. 347, 731–736.
Koning, F.A., Badhan, A., Shaw, S., Fisher, M., Mbisa, J.L., Cane, P.A., 2013. Dynamics of HIV type 1 recombination following superinfection. AIDS Res. Hum. Retrovir. 29, 963–970.
Kraft, C.S., Basu, D., Hawkins, P.A., Hraber, P.T., Chomba, E., Mulenga, J., Kilembe, W., Khu, N.H., Derdeyn, C.A., Allen, S.A., Manigart, O., Hunter, E., 2012. Timing and source of subtype-C HIV-1 superinfection in the newly infected partner of Zambian couples with disparate viruses. Retrovirology 9, 22.
Li, B., Decker, J.M., Johnson, R.W., Bibollet-Ruche, F., Wei, X., Mulenga, J., Allen, S., Hunter, E., Hahn, B.H., Shaw, G.M., Blackwell, J.L., Derdeyn, C.A., 2006. Evidence for potent autologous neutralizing antibody titers and compact envelopes in early infection with subtype C human immunodeficiency virus type 1. J. Virol. 80, 5211–5218.
Moore, P.L., Ranchobe, N., Lambson, B.E., Gray, E.S., Cave, E., Abrahams, M.R., Bandawe, G., Mlisana, K., Abdool Karim, S.S., Williamson, C., Morris, L., Study, C., Immunology, N.C.f.H.A.V., 2009. Limited neutralizing antibody specificities drive neutralization escape in early HIV-1 subtype C infection. PLoS Pathog. 5, e1000598.
Parren, P.W., Marx, P.A., Hessell, A.J., Luckay, A., Harouse, J., Cheng-Mayer, C., Moore, J.P., Burton, D.R., 2001. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. J. Virol. 75, 8340–8347.
Pauthner, M.G., Nkolola, J.P., Havenar-Daughton, C., Murrell, B., Reiss, S.M., Bastidas, R., Prevost, J., Nedellec, R., von Bredow, B., Abbink, P., Cottrell, C.A., Kulp, D.W., Tokatlian, T., Nogal, B., Bianchi, M., Li, H., Lee, J.H., Butera, S.T., Evans, D.T., Hangartner, L., Finzi, A., Wilson, I.A., Wyatt, R.T., Irvine, D.J., Schief, W.R., Ward, A.B., Sanders, R.W., Crotty, S., Shaw, G.M., Barouch, D.H., Burton, D.R., 2019. Vaccine-induced protection from homologous tier 2 SHIV challenge in nonhuman primates depends on serum-neutralizing antibody titers. Immunity 50, 241–252 e246.
Powell, R.L., Kinge, T., Nyambi, P.N., 2010. Infection by discordant strains of HIV-1 markedly enhances the neutralizing antibody response against heterologous virus. J. Virol. 84, 9415–9426.
Ramos, A., Hu, D.J., Nguyen, L., Phan, K.O., Vanichseni, S., Promadej, N., Choopanya, K., Callahan, M., Young, N.L., McNicholl, J., Mastro, T.D., Folks, T.M., Subbarao, S., 2002. Intersubtype human immunodeficiency virus type 1 superinfection following seroconversion to primary infection in two injection drug users. J. Virol. 76, 7444–7452.
Ronen, K., Dingens, A.S., Graham, S.M., Jaoko, W., Mandaliya, K., McClelland, R.S., Overbaugh, J., 2017. Comprehensive characterization of humoral correlates of human immunodeficiency virus 1 superinfection acquisition in high-risk Kenyan women. EBioMedicine 18, 216–224.
Ronen, K., Richardson, B.A., Graham, S.M., Jaoko, W., Mandaliya, K., McClelland, R.S., Overbaugh, J., 2014. HIV-1 superinfection is associated with an accelerated viral load increase but has a limited impact on disease progression. AIDS 28, 2281–2286.
Serwanga, J., Ssemwanga, D., Muganga, M., Nakiboneka, R., Nakubulwa, S., Kiwuwa-Muyingo, S., Morris, L., Redd, A.D., Quinn, T.C., Kaleebu, P., Group, H.I.V.S.S., 2018. HIV-1 superinfection can occur in the presence of broadly neutralizing antibodies. Vaccine 36, 578–586.
Sheward, D.J., Marais, J., Bekker, V., Murrell, B., Eren, K., Bhiman, J.N., Nonyane, M., Garrett, N., Woodman, Z.L., Abdool Karim, Q., Abdool Karim, S.S., Morris, L., Moore, P.L., Williamson, C., 2018. HIV superinfection drives de novo antibody responses and not neutralization breadth. Cell Host Microbe 24, 593–599 e593.
Smith, D.M., Richman, D.D., Little, S.J., 2005a. HIV superinfection. J. Infect. Dis. 192, 438–444.
Smith, D.M., Wong, J.K., Hightower, G.K., Ignacio, C.C., Koelsch, K.K., Petropoulos, C.J., Richman, D.D., Little, S.J., 2005b. HIV drug resistance acquired through superinfection. AIDS 19, 1251–1256.
Smith, S.A., Derdeyn, C.A., 2015. A pathway to HIV-1 neutralization breadth. Nat. Med. 21, 1246–1247.
Tomaras, G.D., Haynes, B.F., 2014. Advancing toward HIV-1 vaccine efficacy through the intersections of immune correlates. Vaccines 2, 15–35.
Trask, S.A., Derdeyn, C.A., Fideli, U., Chen, Y., Meleth, S., Kasolo, F., Musonda, R., Hunter, E., Gao, F., Allen, S., Hahn, B.H., 2002. Molecular epidemiology of human immunodeficiency virus type 1 transmission in a heterosexual cohort of discordant couples in Zambia. J. Virol. 76, 397–405.
van der Kuyl, A.C., Cornelissen, M., 2007. Identifying HIV-1 dual infections. Retrovirology 4, 67.
Wagner, G.A., Landais, E., Caballero, G., Phung, P., Kosakovsky Pond, S.L., Poignard, P., Richman, D.D., Little, S.J., Smith, D.M., 2017. Intrasubtype B HIV-1 superinfection correlates with delayed neutralizing antibody response. J. Virol. 91.
Wall, K.M., Kilembe, W., Vwalika, B., Haddad, L.B., Hunter, E., Lakhi, S., Chavuma, R., Htee Khu, N., Brill, I., Vwalika, C., Mwananyanda, L., Chomba, E., Mulenga, J., Tichacek, A., Allen, S., 2017a. Risk of heterosexual HIV transmission attributable to sexually transmitted infections and non-specific genital inflammation in Zambian discordant couples, 1994-2012. Int. J. Epidemiol. 46, 1593–1606.
Wall, K.M., Kilembe, W., Vwalika, B., Haddad, L.B., Lakhi, S., Onwubiko, U., Htee Khu, N., Brill, I., Chavuma, R., Vwalika, C., Mwananyanda, L., Chomba, E., Mulenga, J., Tichacek, A., Allen, S., 2017b. Sustained effect of couples' HIV counselling and testing on risk reduction among Zambian HIV serodiscordant couples. Sex. Transm. Infect. 93, 259–266.
Williams, K.L., Wang, B., Arenz, D., Williams, J.A., Dingens, A.S., Cortez, V., Simonich, C.A., Rainwater, S., Lehman, D.A., Lee, K.K., Overbaugh, J., 2018. Superinfection drives HIV neutralizing antibody responses from several B cell lineages that contribute to a polyclonal repertoire. Cell Rep. 23, 682–691.
Zanini, F., Brodin, J., Thebo, L., Lanz, C., Bratt, G., Albert, J., Neher, R.A., 2015. Population genomics of intrapatient HIV-1 evolution. Elife 4.