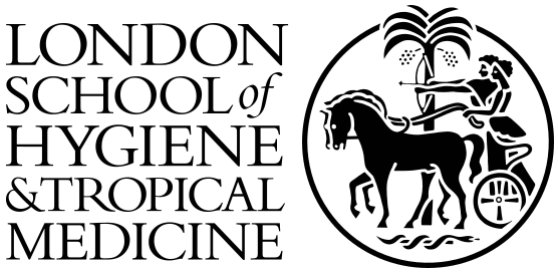

Characterization of the neutralizing antibody response in a case of genetically linked HIV superinfection

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Short Title: Anti-HIV neutralizing antibody response in a linked HIV superinfection

Abstract:

This report describes the identification of a genetically confirmed linked heterosexual HIV superinfection (HIV-SI) where a chronically HIV-infected woman acquired a second strain of the virus from her husband. Serum neutralizing antibody (NAb) responses were examined before and after the HIV-SI, in both the woman and her husband, against their homologous and heterologous viruses including the superinfecting strain. The woman displayed a moderately potent and broad anti-HIV NAb response prior to superinfection, but did not possess NAb activity against the superinfecting strain. This case highlights the unique potential of linked HIV-SI studies to examine natural protection from HIV infection.

Background:

HIV-superinfection (HIV-SI) occurs when an HIV-infected individual acquires a new HIV strain that is phylogenetically distinct from their existing viral population[1]. The majority of studies examining HIV-SI have examined high-risk populations; however, HIV-SI also occurs at significant rates in the general HIV-infected population[1-4]. Screening for cases of HIV-SI in large population cohorts has allowed for the examination of immunological characteristics that may be associated with protection against HIV-SI by comparing superinfection cases to matched HIV-infected controls who do not become superinfected. Of particular interest in these studies has been the potential role of pre-existing HIV-specific neutralizing antibodies (NAb) in protecting against HIV-SI. Two matched case control studies observed that individuals who became superinfected appear to have lower NAb responses compared to controls, but a larger study of female bar workers in Kenya found no association between pre-existing NAb and protection from HIV-SI[5-9]. An alternative approach for exploring HIV-SI risk is to examine HIV-infected couples who acquired their viruses from different sources, thereby making their viral populations phylogenetically unlinked[4, 10]. These couples can then be examined at multiple time points for a linked HIV-SI event if one or both members of the couple pass their virus onto their partner, which then allows for the examination of the underlying immune response to their partner's viral population before and after the HIV-SI event[4, 10, 11].

Methods:

Participants in this study were enrolled in a General Population Cohort (GPC); established in 1989 by the then MRC Programme on AIDS in rural Southwest Uganda (Supplementary Methods)[12]. Individuals in monogamous (n=15) and polygamous relationships (n=6) from the Rural Clinical Cohort in southwest Uganda previously identified as being HIV-infected, but virally unlinked by bulk HIV sequence analysis, were tested for occurrence of HIV-SI by examining longitudinal serum samples for each member of the partnership using a previously described next-generation sequencing (NGS) assay of three viral genomic regions (gag, pol, gp41; Supplementary Methods)[10, 13]. Individuals with

successful NGS of two longitudinal samples for at least one genetic region whose corresponding partner also had NGS data available from the same genetic region were assessed for linked HIV-SI (Table S1). One such event was detected.

For the linked HIV-SI case, serum samples from before and after the time of HIV-SI for both the female, and her male husband, were subjected to single-genome amplification (SGA) in order to generate full-envelope sequences (Fig. S1; Supplementary Methods). For male samples prior to the female's HIV-SI event, full-length SGA was unsuccessful, therefore total RNA was amplified using universal primers and sequenced using a shotgun sequencing method (Supplementary Methods). NGS amplicons specific for the HIV Env gene were matched to the SGA sequences from other time points to verify similarity. Full-length Env amplicons from SGA were subcloned or synthesized and used to generate Env-pseudoviruses. All pseudoviruses were examined for functionality and neutralization susceptibility to known monoclonal antibodies, as well as a variety of subtype A and A/D serum from historic serum samples and non-superinfected Ugandans in the same cohort. Env-pseudoviruses were tested for their neutralization susceptibility to their homologous serum, as well as their partner's heterologous serum from before and after HIV-SI (Supplementary Methods). Viral sequences are available in Genbank (accession numbers MG722983-MG724743).

Results:

Ten individuals had NGS data from at least one genomic region for two time points that matched the same region from their partner's NGS data. Of these ten, one case of a linked HIV-SI was identified. The case occurred in a polygamous relationship in which an HIV-infected uncircumcised male had four wives who were also HIV-positive (Table S1). Longitudinal NGS data was available for both the male and one of his wives from the initial screen (Figure S1). NGS data was available for the pol and gp41 region from only one time point for two of his other three wives, and they were linked to their husband (Figure S2). The fourth wife's virus did not amplify for either time point examined. The male was initially infected approximately four years prior to the HIV-SI event with a recombinant virus that

contained HIV subtype D in the Pol region and subtype A in the gp41 region (Figure 1A and S2). The female was also initially infected approximately four years before the HIV-SI event, and prior to marrying her husband, with a pure subtype A virus in both pol and gp41 (Figure 1A). It was observed that she became superinfected, with a virus that was phylogenetically linked to her husband's viral strain, between 19-22 months after her initial sample (Figure S1). During this three-month period, the woman also became pregnant, and although anti-retroviral therapy (ART) to prevent mother-to-child transmission was not available in this area of Uganda at this time (early 2000s), she later gave birth to a baby that did not become infected with HIV.

Full-length viral Env sequences were obtained from the female partner immediately before HIV-SI (Month 0, n=21) and when HIV-SI was first detected three months later (Month +3, n=10; Figure S4). Three of the viral sequences from this later sample were phylogenetically linked to the male's viruses, thus representing the superinfecting strain (Figure 1B & S4). Full-length envelope sequences from the male partner 12 months after he superinfected the female were also generated (n=24, Figure S4). Full-length Env sequences from the male SGA after HIV-SI contained regions from both subtype A and D, indicating a unique A/D recombinant, and corroborating the assertion that the NGS data from gag and pol came from the same virus (Figure S3). The male had no indication of HIV-SI between the two time points examined by NGS, or in any of the SGA sequences examined later (Figure S2). Repeated attempts to amplify full-length envelopes from earlier time points in the male were unsuccessful; however, shotgun NGS analysis of viral RNA recovered from his serum sample at the time of HIV-SI (Month +0.3) identified one fragment with a 230 bp overlap into the 5' end of the viral envelope. This fragment differed by only one non-synonymous nucleotide mutation from the three superinfecting strains found in the female after HIV-SI (Figure 2C).

Full-length Env amplicons from SGA were subcloned or synthesized and used to generate Env-pseudoviruses for both the female (Month0, n=2; Month +3 n=3 including one SI strain) and male (Month +12, n=9 only four were used for subsequent assays) (Figure 2C and S4)[14]. All pseudoviruses were

examined for functionality and neutralization susceptibility to well-described anti-HIV monoclonal antibodies, as well as a variety of subtype A and A/D serum from historic serum samples and non-superinfected Ugandans in the same cohort[15]. These pseudoviruses demonstrated varying susceptibility to the monoclonal antibodies and serum tested (Figure 2 & S5). Based on this susceptibility, all Env-pseudoviruses from the couple were not unusually sensitive to neutralization, and had a tier-2 like phenotype.

The serum from the female (Months -3,0,+3,+10) and the male (Months +0.3,+12) were tested for their neutralization activity against the couple's Env-pseudoviruses (Figure 2). The female's serum samples prior to HIV-SI displayed moderate NAb activity against her homologous virus. However, her serum prior to HIV-SI and immediately post HIV-SI contained no detectable NAb activity to the superinfecting strain, and weak responses to her husband's strains from one year later (Month +12) that were genetically similar to the superinfecting strain (Figure 2A). Ten months post HIV-SI the female had developed a moderate response to the superinfecting strain (Figure 2). In contrast, there was no increase in NAb response to the other male viruses from Month +12 (Figure 2).

The male's serum at the time of HIV-SI had no detectable neutralizing activity against his wife's strains, and his NAb responses to those strains did not improve one-year post HIV-SI (Figure 2). However, his sera from one year after HIV-SI had high titer NAb activity against the superinfecting strain (Figure 2).

Discussion:

This identification and characterization of a genetically confirmed case of a linked heterosexual HIV-SI event provides a unique opportunity to examine HIV-SI in an individual where her infecting partner is known. In this case, HIV-SI occurred in a chronically infected female who had moderately potent and broad anti-HIV NAb responses. Despite this, she possessed no detectable NAb response to the superinfecting viral strain during the estimated window when HIV-SI occurred, which potentially could

have protected her against the superinfecting strain. This lack of response was not due to an inability to develop a NAb response to this strain since she developed a moderate NAb response to the SI virus approximately seven months after superinfection, as well as a low response to three of four other viruses isolated from her male partner. It is interesting that the male possessed a very limited NAb response to the viruses tested, even after being infected for over 30 months at the time of HIV-SI. However, like his female partner his NAb response to the superinfecting strain, which originally came from him, increased significantly 12 months after the superinfection occurred.

There is a large body of preclinical data indicating that NAb can confer protective immunity against animal lentiviruses. The data from this case report agree with the widely held concept that NAb are an important component of protective immunity against HIV infection, and thus a successful HIV vaccine should aim to elicit a broadly reactive NAb response[16]. As with any single case, these data are supportive, but not conclusive. Also, this study was limited by the sample types (serum only) and volumes available, as this was a secondary analysis of a previous study performed over fifteen years ago. The limited sample volume for this couple precluded examining other interesting aspects of the humoral immune response that may play a role in protection against HIV-SI, as well as limiting the ability to fully characterize the neutralization breadth of the couple before and after HIV-SI. The totality of the data were also limited by the inability to amplify full envelope sequences from the male partner prior to HIV-SI. However, the superinfecting strain's viral envelope sequence isolated from the woman at the time of HIV-SI was almost identical to a fragment of envelope sequence taken from the man prior to HIV-SI, suggesting that this isolate is extremely similar to the superinfecting viral strain.

Notably, the male possessed no detectable NAb response to the female's heterologous virus, yet he did not become superinfected. This could be influenced by the possibility that NAb have no protective role against HIV-SI, the increased risk of male-to-female transmission compared to female-to male, or that he was protected by a different immunological response not examined here[8, 17]. In summary, this

case demonstrates the exciting amount of potential information that even a small number of these types of cases could provide, and supports the need to further examine historic cohorts for linked HIV-SI events.

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Figure 1: Sequencing results demonstrate a linked HIV-SI event: A) Neighbor-joining phylogenetic tree of consensus gp41 viral sequences (≥10 reads) derived from next-generation sequencing (NGS) of the female (Red) and male's (Blue) initial time point (-19 months), as well as the female's viral sequences immediately post HIV-SI (Month +3; Green) with the superinfecting viral strains clustering with the husband's virus. Number of repeated sequences represented by each NGS consensus sequence is shown at the end of the consensus identifier. B) Neighbor-joining tree of full SGA derived viral envelopes used for pseudotyped viruses. C) Neighbor-joining tree of 230 bp of the 5' end of the viral envelope from the pseudotyped viral isolates aligned with the NGS shotgun-sequencing fragment from husband's sample prior to HIV-SI (Orange). The fragment clusters with the superinfecting strain found in his wife immediately after HIV-SI. Distances are indicated for the tree by the scale at bottom, and samples are grouped with a selection of subtype reference sequences (black). Bootstrap values greater than 80 percent are indicated (1000 replicates).

Figure 2: Female member's sera did not neutralize superinfecting viral strain: A) Values in table indicate the dilution of the heat-inactivated serum required to block fifty percent of a standard infectious dose (ID50): weak (green), moderate (yellow), and strong (orange) neutralization values are highlighted. Along the top of the table are indicated time points of female and male sera, as well as a collection of sera from HIV-subtype A and A/D infected individuals. The three columns to the left show information on Env-pseudoviruses tested, including the month and visit time point. The female SI virus (SI-Female Month+3_v2) is shown colored in green. Sera from individuals screened for linked superinfection are indicated by couple number and member ID (Table S1). To provide a benchmark for the varied levels of neutralization activity against autologous viruses, the male and female's sera were also tested against a panel of six previously described HIV-pseudoviruses (Heterologous virus panel). The female's serum samples prior to HIV-SI displayed a measurable NAb response to five of the six unassociated pseudoviruses, and the male's serum at the time of HIV-SI was weakly neutralizing against all the pseudoviruses tested. B) ID50 values of the female's samples over time against the corresponding

194 heterologous and homologous pseudoviruses are shown (Blue-male pseudoviruses, Red-female
195 pseudoviruses, Green-superinfecting strain).

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197 **Supporting Information Legends**

198 Table S1: Successful next-generation sequence screening data for Ugandan couples

199 Figure S1: Timeline of viral load and sequencing of linked HIV-SI event

200 Figure S2: NGS data for linked HIV-SI family.

201 Figure S3: Viral envelope from the male was identified as an A/D recombinant.

202 Figure S4: Neighbor-joining tree of full-length SGA derived viral envelopes from the male at month_+12
203 and from his wife at month_0 and _+3 after the beginning of the superinfection window.

204 Figure S5: Envelopes from male and female have a tier 2-like neutralization phenotype.

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Supplementary Methods

Study Participants:

Participants in this study were enrolled in a General Population Cohort (GPC); established in 1989 by the then Medical Research Council Programme on AIDS in rural Southwest Uganda[1, 2]. The GPC initially enrolled approximately 5,000 adults drawn from a cluster of 15 villages and later in 1990, a random selection of one-third of seropositive adults identified in the initial GPC serosurvey round were invited to enroll into the Rural Clinical Cohort (RCC), previously called Natural History Cohort, as prevalent HIV cases[3]. Thereafter, all new HIV seroconverters were invited to enroll as incident cases. Participants enrolled in the RCC attend the study clinic every three months for clinical history, examination and blood sampling and HIV infected participants are encouraged to bring their partner(s) for voluntary counseling and testing and possible enrollment. This study was approved by the Science and Ethics Committee of the Uganda Virus Research Institute and by the Uganda National Council for Science and Technology. Clinical, epidemiological data and blood samples were obtained following informed consent.

Next-generation sequencing to screen for HIV superinfection

As reported previously, viral RNA was extracted from ~140 µL of the first and last serum samples available for each member of the couples, reverse-transcribed, and amplified in a nested-PCR format for a region of the viral p24 (~390 bp), reverse transcriptase (~530 bp) and gp41 (~324 bp) coding regions[4, 5]. Subject samples that amplified for both time points in at least one region were sequenced using the 454 DNA Sequencing platform (Roche, Branford, CT). Pools of samples were processed using emPCR Amplification Manual-Lib-L-LV–June

2013(Roche Branford, CT) using 25% of the recommended amplification primer amount and a 0.2 copy-per-bead ratio[4].

The resulting sequencing reads were analyzed and similar sequences were combined into a single consensus sequence. Consensus sequences that encompassed a cluster of at least ten individual, near-identical sequence reads were determined and used for all subsequent analyses[4]. All consensus sequences were examined and single consensus sequences that matched the prominent species for another sample in the same plate were removed as contamination. Linked HIV superinfection (HIV-SI) was defined when a subject's follow-up sample demonstrated two or more distinct consensus sequences forming a phylogenetic cluster that was of adequate genetic distance from the baseline sequences to rule out evolutionary drift from the individual's initial consensus sequences, and that was phylogenetically linked to their partner's viral sequences at their first sample time point [4]. The window period of possible HIV-SI was determined by NGS of all available serum samples between the first and last available sample for the superinfected individual.

Single genome amplification (SGA), sequencing and cloning

Single genome amplification (SGA), sequencing, and cloning of HIV-1 envelope genes were performed as reported previously [6]. Briefly, HIV-1 RNA was isolated from serum using the QiaAmp vRNA mini kit (Qiagen). HIV-1 RNA was then reverse transcribed to cDNA using SuperScript III Reverse Transcriptase (Life Technologies) and previously described clade A reverse primers nef 50 (5'-AGAGCTCCCTTGTAAGTCATTGG-3') or nef24 (5'TACTTGTGATTGCTCCATGT-3') or newly synthesized nefvrc2 (5'-CTTTCCCTTATAGCAGGCCATC-3') [6, 7]. Subsequent PCR was performed under limiting

dilution conditions, diluted to yield amplification in no more than 25% of wells. Nested PCR of HIV-1 env was performed with different primer sets as follows. First round PCR was performed either with primers previously described, vpr1 (5'- GATAGATGGAACAAGCCCCAG-3') and nef24 (5'- TACTTGTGATTGCTCCATGT-3') or newly designed primers nefvrc2 (5'- CTTTCCCTTATAGCAGGCCATC-3') and vprvrc1 (5'- CACCTATGGCAGGAAGAAGCGGAG-3') [6, 7]. Thermocycler conditions were 94°C for 2 minutes followed by 35 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 4 seconds with a final incubation at 68°C for 10 minutes. Second round PCR was performed with primers previously described, including vpr21a1 (5'- TAACCTAGACGCGTGGAATCACCCGGGAAGTCAGCCTACAACACCTTGTA -3'), vpr21a2 (5'- TAACCTAGACGCGTGGAATCACCCGGGAAGCCGGCCTACAACACCTTGTA-3'), nef60a1 (5'- CTTGTGGCGGCCGCATGTTTATCTAAATCTCGAGATACTGCTCCTACTCCTGGTGCTG -3'), and nef60a2 (5'- CTTGTGGCGGCCGCATGTTTAGCTAAATCTCGAGATACTGCTCCTACTCCTGCTGCTG -3'), or newly designed primers including vprvrc5 (5'- CACCAATAAGAGAAAGAGCAGAAGACAG-3') and nefvrc5 (5'- CTATRCTACTTTTTGACCACTTG-3') [8]. Thermocycler conditions were 94°C for 2 minutes followed by 45 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 4 seconds with a final incubation at 68°C for 10 minutes. The amplicons from envelope genes from single genome templates were directly sequenced by ACGT, Inc. (Germantown, MD). The full-length envelope sequences were assembled and edited using Geneious software.

Envelope amplicons were cloned into the pcDNA 3.1 vector (directional) (Life Technologies) by re-amplification of SGA first round products using Phusion DNA polymerase (Agilent Technologies) with primers vprvrc1 (5'- CACCTATGGCAGGAAGAAGCGGAG-3') and nefvrc5 (5'-CTATRCTACTTTTTGACCACTTG-3'). Cloned env genes were sequenced to confirm that they exactly matched the sequenced amplicon.

SGA was successful for the female samples (0, +3 Months) and the male sample (+12 Months). Of note, no amplicons were obtained from four earlier samples from the male (three time points pre-SI window and one in the SI-window). Three envelope sequences from the female that were amplified, but could not be subcloned (SI-Female Month+3_v1, SI-Female Month+3_v2, and SI-Female Month+3_v9) were codon optimized, synthesized, and then subcloned (GenScript, Piscataway, NJ). Envelope clones were used to generate single round of replication Env-pseudoviruses, as described below.

The subtype of the Env sequences was determined using the RIP program via the website https://www.hiv.lanl.gov/content/sequence/RIP/RIP.html [9]. The male Env sequences were determined to be A/D recombinant, with 4 breakpoints in the rev-env region, using the RIP tool as well as analysis by jumping profile Hidden Markov Model via the website http://jphmm.gobics.de/jphmm.html [10].

Shotgun sequencing

For male samples (-18, +0.3 Months), total RNA was extracted from serum using RNAzol RT (Molecular Research Center, Inc, Cincinnati, OH), according to the manufacturer's protocol. RNA was fragmented, reversed transcribed using random hexamers and Illumina-ready libraries were generated based on Illumina's TruSeq platform. The Illumina-ready libraries were sequenced by paired-end MiSeq 2x150 base pair reads. HIV contigs were generated by

performing *de novo* assembly with the short-read transcript assembler, Trinity, from the Broad Institute. The assembled contigs were then aligned against the "nt" database from NCBI using BLAST+. Contigs that aligned to HIV reference sequences were extracted for further analysis. Only the +0.3 Months sample yielded a fragment that matched HIV sequence, corresponding to nt 6184-6453 in the genome (HxB standard) and spanning the 3' end of *vpu* and the 5' end of *env*.

Pseudovirus production

As described previously, env-pseudoviruses were produced by co-transfecting 293T cells with cloned viral envelope plasmids and a full length HIV clone with envelope deleted (SG3Δenv)[11]. The pseudoviruses were characterized with a panel of 10 mAbs in the TZM-bl neutralization assay (described below), including VRC01, PGT121, PGT128, PGT145, 10E8, CAP256-VRC26.25, N123-VRC43.01, 17b, 447-52D, and F105. The env-pseudoviruses from the female and male samples were used to test the level of NAb neutralization from the male and female serum samples.

A 6-virus heterologous panel was generated to test NAb breadth in the serum of the male and female. This panel included BG505.W6M.C2, KER2018.11, Q842.d12, Q461.e2, TRO.11, and DU422.01.

Neutralization assays

Neutralization assays were performed as previously described[11, 12]. Briefly, pseudovirus was mixed with serial dilutions of serum or monoclonal antibodies, incubated together for 30 minutes, and then added to TZM-bl target cells which express luciferase upon infection. The data were calculated as a reduction in luminescence units compared with control wells, and reported

as 50% inhibitory concentration (IC_{50}) in micrograms per microliter for monoclonal antibodies, or 50% inhibitory dilution (ID_{50}) for serum.

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Supplementary Tables and Figure Legends

Table S1: Successful next-generation sequence screening data for Ugandan couples

Couple Number	Partner idenity	Person years of follow-up	Gag subtype	Gag SI	Pol subtype	Pol SI	gp41 subtype	gp41 SI	Final SI status
1	M	2.76	A	No	A	No	A	No	
1	F-1	N/A	-	-	-	-	-	-	
2	M	1.50	D	-	-	-	-	-	
2	F-1	3.02	D	-	-	-	-	-	
2	F-2	1.50	C	-	-	-	-	-	
4	M	2.02	A	No	-	-	A	No	
4	F-1	1.02	-	-	-	-	A	-	
5	M	2.94	-	-	D	-	M (D/A)	-	
5	F-1	3.76	-	-	D	No	A	No	
5	F-2	3.75	-	-	A	-	M (D/D)	-	
5	F-3	5.48	-	-	D	-	A	-	
8	M	1.06	D	No	D	No	D	No	
8	F-1	2.47	D	No	D	No	D	No	
9	M	3.07	M (A/A)	No	A	No	A	Yes	Not linked SI
9	F-1	3.93	A	-	A	-	A	-	
9	F-2	6.32	-	-	-	-	-	-	
9	F-3	2.90	A	-	A	-	A	-	
10	M	N/A	D	-	D	-	A	-	
10	F-1	0.00	A	No	A	No	A	No	
11	M	5.51	-	-	-	-	A	No	
11	F-1	5.05	-	-	-	-	A	-	
12	M	1.19	-	-	A	-	-	-	
12	F-1	2.00	A	No	D	No	D	No	
14	M	0.95	D	-	D	No	D	-	
14	F-1	1.04	D	-	D	-	D	-	
15	**M**	**6.24**	**-**	**-**	**D**	**No**	**A**	No	
15	**F-1**	**1.54**	**-**	**-**	**D**	**-**	**A**	**-**	
15	**F-2**	**4.99**	**D**	**-**	**D**	**-**	**A**	**-**	
15	**F-3**	**2.49**	**-**	**-**	**A**	**-**	**A**	**Yes**	**Linked SI**
15	**F-4**	**2.76**	**-**	**-**	**-**	**-**	**-**	**-**	
17	M	2.90	-	-	-	-	-	-	
17	F-1	3.77	-	-	D	No	D	No	
21	M	6.52	D	-	D	-	-	-	
21	F-1	0.27	A	No	D	No	-	-	

-Male (M) and female (F) members of the couples are indicated; subtypes are indicated and were determined phylogenetically (M=multiple infections detected with subtypes of each variant population identified); (-) in the subtype column indicates that NGS for that genetic region was unsuccessful for that subject; (-) in the SI column indicates only one time point was successful for that genetic region. NGS was not successful for either member of couples 3, 6, 7, 13, 16, 18, 19, and 20. The male from couple 9 had evidence of a HIV-SI in the gp41 region. The new superinfecting strain was not phylogenetically linked to any of his female partners, and therefore was determined to be unlinked.

Figure Legends

Figure S1: Timeline of viral load and sequencing of linked HIV-SI event: The female's (red) and male's (blue) viral loads are indicated prior to and after the HIV-SI window (yellow box). Samples where next-generation sequences were obtained are indicated by arrows (Red, Female; Blue, Male). Samples where single genome amplification (SGA) sequencing was also successful are shown by arrows with solid fill, and the number of resulting functional pseudoviruses used for neutralization assays are indicated in boxes above (Green indicates superinfecting strain).

Figure S2: **NGS data for linked HIV-SI family.** A) Neighbor-joining phylogenetic tree of consensus gp41 viral sequences (≥10 reads) derived from next-generation sequencing (NGS) of the male's (Blue) initial time point (-19 months) and his follow-up time point (+56 months; Purple). B) Neighbor-joining phylogenetic tree is shown including the male (Blue) and his wives' initial gp41 NGS viral sequences (Female 1-Pink; Female 2-Orange, and Female 3-Red). C) Neighbor-joining phylogenetic tree is shown including both male sample time points (-19 months-Blue, and +56 months-Purple) and his wives' pol NGS viral sequences (Female 1 +54 months-Pink; Female 2 +9 months-Orange, and Female 3 -19 months-Red). Number of repeated sequences represented by each NGS consensus sequence is shown at the end of the consensus identifier. Distances are indicated for the tree by the scale at bottom, and samples are grouped with a selection of subtype reference sequences (black).

Figure S3: Viral envelope from the male was identified as an A/D recombinant. Subtype determination and breakpoint locations are shown and were calculated using jpHMM-HIV. Base pair locations are shown above and refer to HXB2 genome reference sequence. One representative sequence is shown.

Figure S4: **Neighbor-joining tree of full-length SGA derived viral envelopes from the male at month_+12 and from his wife at month_0 and _+3 after the beginning of the superinfection window**. Sequences that either could not be cloned (_nC), weren't entry competent (_nE), or were not made into pseudoviruses (_nP) are indicated at the end of the sequence name. Sequences from the male

that were made into fully functional pseudoviruses are shown (blue) with sequences not made into pseudoviruses also shown (light blue). Similarly, the wives' primary viruses (red and pink), and superinfecting strains (green and light green) are shown. Distances are indicated for the tree by the scale at bottom, and samples are grouped with a selection of subtype reference sequences (black).

Figure S5: **Envelopes from male and female have a tier 2-like neutralization phenotype**. Neutralization susceptibility to monoclonal antibodies for pseudoviruses derived from the male and female couple members. Heat map showing the concentration of the neutralizing antibody required to block fifty percent of a standard infectious dose (IC50). Pseudoviruses used for other assays are shown in bold.

Figure 1:

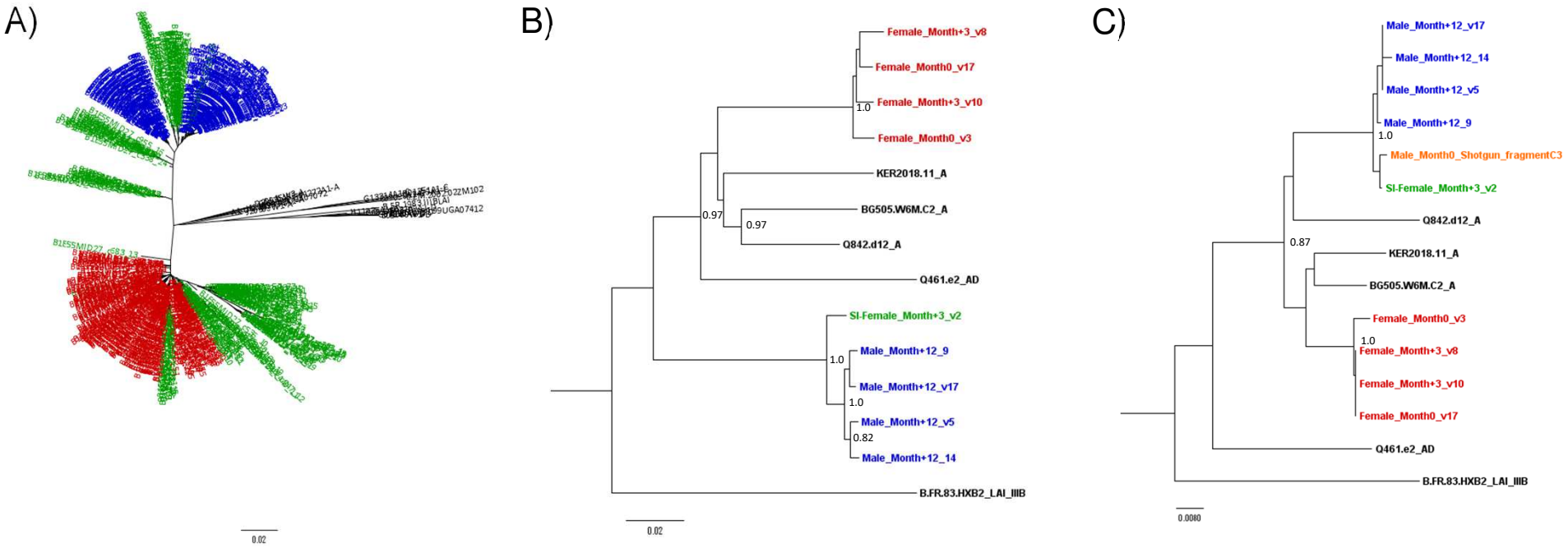
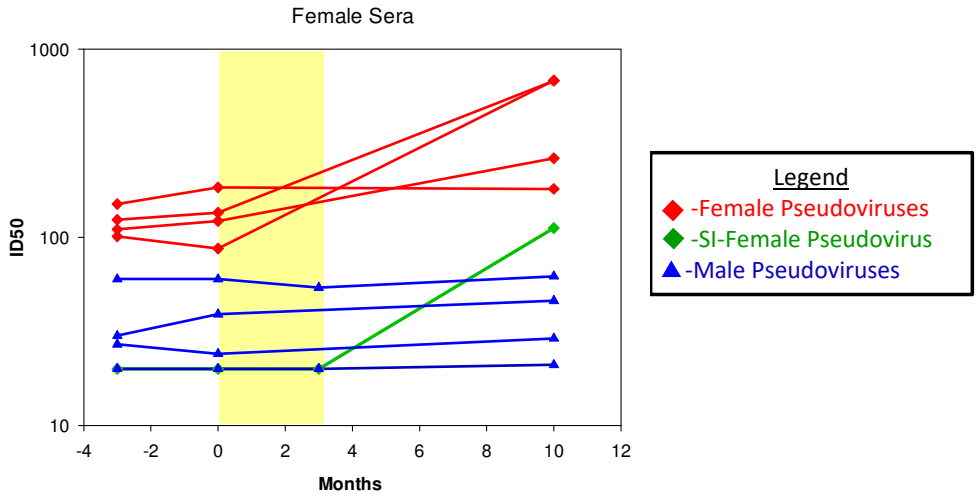

A)
B)
Female_Month+3_v8
Female_Month0_v17
Female_Month+3_v10
Female_Month0_v3
KER2018.11_A
BG505.W6M.C2_A
Q842.d12_A
Q461.e2_AD
SI-Female_Month+3_v2
Male_Month+12_9
Male_Month+12_v17
Male_Month+12_v5
Male_Month+12_14
B.FR.83.HXB2_LAI_IIIB
1.0
0.97
0.97
1.0
1.0
0.82
0.02
C)
Male_Month+12_v17
Male_Month+12_14
Male_Month+12_v5
Male_Month+12_9
Male_Month0_Shotgun_fragmentC3
SI-Female_Month+3_v2
Q842.d12_A
KER2018.11_A
BG505.W6M.C2_A
Female_Month0_v3
Female_Month+3_v8
Female_Month+3_v10
Female_Month0_v17
Q461.e2_AD
B.FR.83.HXB2_LAI_IIIB
1.0
0.87
1.0
0.0080

Figure 2:

A)

			Female Sera				Male Sera		Clade A/AD Sera Controls								
Virus from Sample	Pseudovirus	Subtype	-3 Months	0 Months	+3 Months	+10 Months	+0.3 Months	+ 12 Months	CHAVI219 2008	POC36633 1995	POC44951 1996	APOS101 2004	Uganda 357139	Uganda 191520	Uganda 119601	Uganda 355472	Uganda 171950
Female 0 Months	Female_Month0_v3	A	101	87	ND	681	<20	<20	102	<20	44	<20	69	72	112	62	78
	Female_Month0_v17	A	124	135	ND	680	<20	<20	101	164	158	<20	124	112	180	121	108
Female +3 Months	Female Month+3_v8	A	110	122	ND	263	<20	<20	72	68	199	<20	105	80	129	90	85
	Female Month+3_v10	A	150	184	ND	181	<20	<20	51	43	84	<20	91	79	100	95	62
	SI-Female Month+3_v2	AD	<20	<20	<20	112	34	870	410	<20	<20	<20	<20	22	28	<20	<20
Male +12 Months	Male Month+12_v5	AD	60	60	54	62	60	125	782	<20	<20	35	<20	104	102	<20	<20
	Male Month+12_v9	AD	30	39	ND	46	26	44	904	<20	<20	33	<20	123	153	<20	<20
	Male Month+12_v14	AD	<20	<20	<20	21	<20	44	478	<20	<20	36	<20	35	52	<20	<20
	Male Month+12_v17	AD	27	24	ND	29	21	53	950	<20	<20	24	<20	143	271	<20	<20
Heterologous Virus Panel	BG505.W6M.C2	A	62	35	ND	29	ND	29	721	<20	<20	62	30	<20	71	<20	<20
	KER2018.11	A	143	230	ND	294	ND	24	351	<20	<20	55	26	<20	23	<20	20
	Q842.d12	A	344	68	ND	344	ND	29	1240	79	116	36	151	109	211	101	137
	Q461.e2	AD	<20	<20	ND	<20	ND	<20	99	<20	<20	<20	<20	<20	<20	<20	<20
	TRO.11	B	31	29	ND	34	ND	96	218	<20	<20	140	<20	33	58	<20	<20
	DU422.01	C	166	27	ND	274	ND	25	77	111	116	45	121	74	151	76	87

B)



Legend
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20-100
<20