Vaccine 36 (2018) 578–586

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

HIV-1 superinfection can occur in the presence of broadly neutralizing antibodies

Jennifer Serwanga [a,*], Deogratius Ssemwanga [a], Michael Muganga [a], Ritah Nakiboneka [a], Susan Nakubulwa [a], Sylvia Kiwuwa-Muyingo [a], Lynn Morris [b,c,h], Andrew D. Redd [d,e], Thomas C. Quinn [d,e], Pontiano Kaleebu [a,f], The HIV Superinfection Study Group [1]

[a] MRC/UVRI Uganda Research Unit on AIDS, c/o Uganda Virus Research Institute, Entebbe, Uganda
[b] National Institute for Communicable Diseases, Johannesburg, South Africa
[c] Center for the AIDS Program of Research in South Africa (CAPRISA), South Africa
[h] University of the Witwatersrand, Johannesburg, South Africa
[d] Laboratory of Immunoregulation, Division of Intramural Research, NIAID, NIH, Bethesda, USA
[e] Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA
[f] London School of Hygiene and Tropical Medicine, Department of Clinical Research, London, UK

ARTICLE INFO

Article history:
Received 11 July 2017
Received in revised form 28 October 2017
Accepted 26 November 2017
Available online 20 December 2017

Keywords:
HIV-1
Superinfection
Clade A
Clade D
Broadly neutralizing antibodies

ABSTRACT

Background: Superinfection of individuals already infected with HIV-1 suggests that pre-existing immune responses may not adequately protect against re-infection. We assessed high-risk female sex workers initially infected with HIV-1 clades A, D or A/D recombinants, to determine if HIV-1 broadly neutralizing antibodies were lacking prior to superinfection.

Methods: Six superinfected female sex workers previously stratified by HIV-1 high-risk behavior, infecting virus clade and volunteer CD4 counts were evaluated at baseline (n = 5) and at 350 days post-superinfection (n = 6); one superinfected volunteer lacked pre-superinfection plasma. Retrospective plasmas were assessed for neutralization of a multi-clade panel of 12 HIV-1 viruses before superinfection, and then at quarterly intervals thereafter. Similarly stratified singly infected female sex workers were correspondingly assessed at baseline (n = 19) and 350 days after superinfection (n = 24). Neutralization of at least 50% of the 12 viruses (broad neutralization), and geometric means of the neutralization titers (IC_{50}) were compared before and after superinfection; and were correlated with the volunteer HIV-1 superinfection status, CD4 counts, and pseudovirus clade.

Results: Preexisting broad neutralization occurred in 80% (4/5) of the superinfected subjects with no further broadening by 350 days after superinfection. In one of the five subjects, HIV-1 superinfection occurred when broad neutralization was lacking; with subsequent broadening of neutralizing antibodies occuring within 9 months and plateauing by 30 months after detection of superinfection. Clade B and C pseudoviruses were more sensitive to neutralization (13; [87%]); and (12; [80%]) than the locally circulating clades A (10; [67%]) and D (6; [40%]), respectively (p = 0.025). Low antibody titers correlated with clade D viruses and with >500 CD4 T cell counts, but not with the superinfection status.

Conclusion: These data demonstrate that HIV-1 superinfection can occur both in the presence, and in the absence of broadly neutralizing antibodies.

1. Introduction

HIV-1 infected individuals who regularly practice unprotected sex with multiple sexual partners are prone to superinfection with new HIV-1 strains. This has been more frequently reported in early HIV-1 infection when host responses are relatively lower [1,2], implying that the natural anti-HIV immune responses may fail to prevent superinfection. This concept is supported by studies that associated superinfected individuals with lower broadly neutralizing antibody levels than their singly infected counterparts [2]. However, superinfection has also been shown in chronic HIV-1 disease where host immune responses are better established [3,4]. This suggests that pre-existing antibodies may not always adequately prevent further infection.

* Corresponding author.
E-mail address: Jennifer.Serwanga@mrcuganda.org (J. Serwang).
[1] See complete list of HERO consortium co-authors in the online version.

https://doi.org/10.1016/j.vaccine.2017.11.075
0264-410X/

Eliciting protective anti-HIV-1 antibodies is hindered by the continuous mutation of HIV-1 generating extensive diversity and eventual escape from the existing antibody response. Many monoclonal antibodies with significant coverage of the broad HIV-1 diversity have been isolated [5–8]. These prevented mucosal in macaques and humanized mice [9–12], and led to varying control of viraemia in human [13–15] and animal models [16–18], especially when used in combination [17]. Likewise, the subsequent 50% reduction in risk for HIV-1 infection of infected humans suggested potential protectiveness of pre-existing immunity [19]. HIV-1 superinfection is therefore as a good model for evaluating correlates of immune protection from HIV-1 infection. Here, we took advantage of existing stored plasmas from an incident female superinfected sex worker cohort [20] to evaluate relationships between neutralizing antibody responses and occurrence of HIV-1 superinfection.

2. Materials and methods

2.1. Study population

Quarterly plasmas from adult female sex worker participants of a high-risk HIV-1 behavioral cohort were collected between May 2008 and Dec-2013 [21,22]. Next-generation sequencing of partial p24 *gag* and *gp*41 envelope HIV genomic regions was used to screen the plasmas for HIV-1 superinfection, as described elsewhere [20,23]. Participants were categorized by the initial infecting HIV clade, HIV-1 risk behavior, and last CD4 T Cell count prior to superinfection. Trends of participants CD4 T cell counts over time were computed. Participant Superinfection timing was estimated as the median time between the last plasma with a single infecting strain and the first plasma with a newly acquired HIV-1 strain.

Of 85 screened drug-naïve female sex workers, seven with confirmed superinfection were evaluated for availability of stored specimens and screened for HIV-1 neutralizing antibodies at baseline (n = 5) and 350 days after study entry (n = 6). Singly infected participants were similarly assessed at baseline (n = 19) and at day 350 (n = 24). Ethical approval to conduct these studies was obtained from Uganda Virus Research Institute (UVRI) Research and Ethics Committee (REC), and from the Uganda National Council for Science and Technology (UNCST).

2.2. Preparation of pseudoviruses

Molecularly cloned pseudotyped viruses were generated in 293 T cells by co-transfection of specified envelope (*Env*) plasmids with *Env*-defective backbone plasmids (pSG3ΔEnv), and titrated in TZM-bl cells, as described elsewhere [24,25]. Plasmids were obtained from Prof Lynn Morris' lab, and from the National Institutes of Health AIDS Research and Reference Reagent Program. Neutralisation of a twelve-virus panel of tier II viruses comprising clade A, (Q23.17; Q769.d22 and Q842.d12); clade B (TRO.11, 6535.3 and RHPA); clade C (ConC; CAP45.G3 and Du156), and D viruses (QA013.H1; QB857.B3; and QD435.B5) was assessed. Where specimen volumes allowed, the effect of substitutions N160K and N33A at the envelope glycan sites known to be critical for broadly neutralization was evaluated to map specificities of the neutralizing antibody response.

2.3. Neutralization assays

Reduction in virus infection of target cells was quantified using Tat-regulated firefly luciferase reporter gene expression, as described elsewhere [25,26]. Briefly, duplicate wells of heat-inactivated, threefold serially diluted plasmas ranging from 1:40 to 1:1080 were screened for neutralization of a 12-virus panel. Wells with cells and media only were used for quantifying background luminescence. Virus controls were wells containing virus-infected cells, but lacking test plasmas. Wells with a strain of Vesicular Stomatitis Virus G (SVA.MLV) that is unrelated to HIV were included to screen out non-specific reactivity, and potential exposure to anti-retroviral drugs. Positive controls were plasmas predetermined to neutralize specified viruses.

Neutralization of HIV-1 was determined from linear interpolation of plotted virus infectivity curves as 50% inhibitory titres (IC_{50}). Inhibitory titres were computed as reciprocals of plasma dilutions that reduced relative luminescence in test wells by 50% compared to that in virus control wells. The assay lower detection limit was 40; therefore, titres below 40 were considered undetectable and were arbitrarily assigned to be 20 for ease of analysis. Inhibitory titres of 40 and above were confirmed in a repeat test, and an average titre computed. Plasma with titres of 1080 and above were retested in threefold serial dilution ranging from 1:20 to 1:43,740, to define the endpoint titre. All neutralization titres used are derived from means of two independent tests.

Neutralization breadth was determined as the proportion (%) of the 12 viruses inhibited. Stricter criterion was used to determine titres for neutralizing breadths, inhibitory titres below 50 were considered unreactive. Plasmas that neutralized at least 50% of the viruses were considered to be broadly neutralizing. Geometric means of the neutralization breadths and neutralization titres were correlated with SI status, CD4 counts, and infecting virus clade.

2.4. Statistical analyses

Statistical analyses and graphical presentations were performed using Graph Pad 5.0 (GraphPad Software, Inc., San Diego, California USA). Neutralization breadths and geometric means of the titers (IC_{50} values) were compared at two time points: (i) pre-superinfection, as the closest time point to the visit at which superinfection was detected, and (ii) 350 days post-superinfection, as the earliest analysis time point after superinfection. Median matched values were compared using the Wilcoxon rank sum test. Geometric means of titres were compared using t-tests. Regression analyses and confidence intervals of neutralizing antibody means (across 12 viruses) were computed over time.

Regression analysis was also used to estimate the overall CD4 + T Cell slopes over time. Slope estimates were expressed as coefficients. Negative coefficients indicated decreasing trend of neutralizing antibodies/CD4 T Cell counts over time, and positive slopes indicate increasing trend over time. P-values of ≤ 0.05 indicated that the slope was significantly different from zero. Factors linked to SI status relative to one's CD4 count strata and HIV-1 risk behavior profiles (number of sexual partners) were assessed using a conditional logistic regression model. A normal interval regression model of generalized estimating equations was used to evaluate effects of SI status on absolute log10 inhibitory titres, after adjusting for CD4 counts, pre-SI breadth, neutralization titers and infecting clade.

3. Results

3.1. Study participants

We present profiles of HIV neutralizing antibody responses for six superinfected, and 24 singly infected drug naïve female sex workers. Median participation time since study entry for all six superinfected cases was 313 days (Interquartile range [IQR] 295–367). One superinfected volunteer lacked plasma prior to superinfection; thus, their pre-superinfection data constitutes five

superinfected cases. Baseline CD4 counts were ≥500 cells/μl in three superinfected cases (Fig. 1C, E and F), and <500 cells/μl for the rest (Fig. 1A, B and D). Participant CD4 T cells were significantly declining over time in three superinfected (Fig. 1B, D and F, $p \leq 0.05$) and six singly infected individuals (Fig. 1A, B and D). Participant CD4 T Cell counts did not differ over time in three superinfected (Fig. 1A, C and E) and 16 singly infected individuals (Fig. 1A–F). Participant CD + T Cell counts significantly increased over time in two singly infected individuals (Fig. 1E and F). Initial infecting virus strains (*gp*41 and *gag* p24 genomic regions) were either clades A, D or recombinant A/D, summarized in Fig. 1.

3.2. Superinfection occurred in the presence of HIV-specific broadly neutralizing antibodies

All analysed plasmas lacked non-specific HIV-1 inhibition as evaluated using the non-HIV pseudovirus control (SVA-MLV). All HIV-1 negative plasmas (negative controls) lacked inhibitory activity against the evaluated pseudoviruses. Prior to superinfection, broad neutralization was present in four of the five superinfected individuals, with distinct patterns of within-clade neutralization clustering, suggestive of differential sensitivity to neutralization across strains. Clade B and C viruses that are not prevalent in this population were more sensitive [(13 of 15 tests; [87%]) and (12 of 15; [80%]) than the endemic clade A (10 of 15; [67%]) and D (6 of 15; [40%]), respectively, p = 0.025, Fisher's Exact test, Fig. 2A.

A multivariable model assessing relationships between superinfection and ensuing antibody titers from baseline revealed independent associations with CD4 T cell counts at preceding visits and ability to neutralize clade D viruses ($p < 0.001$), but not with SI status (p = 0.6), Table 1. A conditional logistic regression model stratifying by infecting clade showed significantly narrower clade D breadths than clade A, B and C, $p < 0.001$, Table 1. The study sample size was too small to infer reliable statistical interpretations. However, clade D tended to exhibit lower antibody titres (median 41, Interquartile range [IQR] 24–55) than A (111, IQR 31–244), B (84, IQR 56–263) and C (125, 77–201), p = 0.08, one-way ANOVA, Fig. 2B. Lower neutralizing antibody titres were also associated with CD4 T Cell counts greater than 500 cells/μl. Of the assessed clade A, B, C and D viruses, strains Q769.d22, RHPA, CAP45.G3 and QD835.B3 were the least sensitive within each clade, respectively.

Approximately 350 days after superinfection, three of six (50%) superinfected participants had developed detectable virus-specific broadly neutralizing antibodies (Fig. 2C). Proportions of clade tests with clade D sensitivity (8 of 18; 44%) did not significantly differ from those with sensitivity to clade A (14 of 18; 78%), B (14 of 18; 78%) and C viruses (12 of 18; 67%), $p > 0.05$ respectively, Fisher's Exact test, Fig. 2C. Similar to what was seen during the pre superinfection stage, clade D tended to have lower titres (median 33, Interquartile range [IQR] 25–62) than clade A (127, IQR 33–237), B (236, IQR 36–

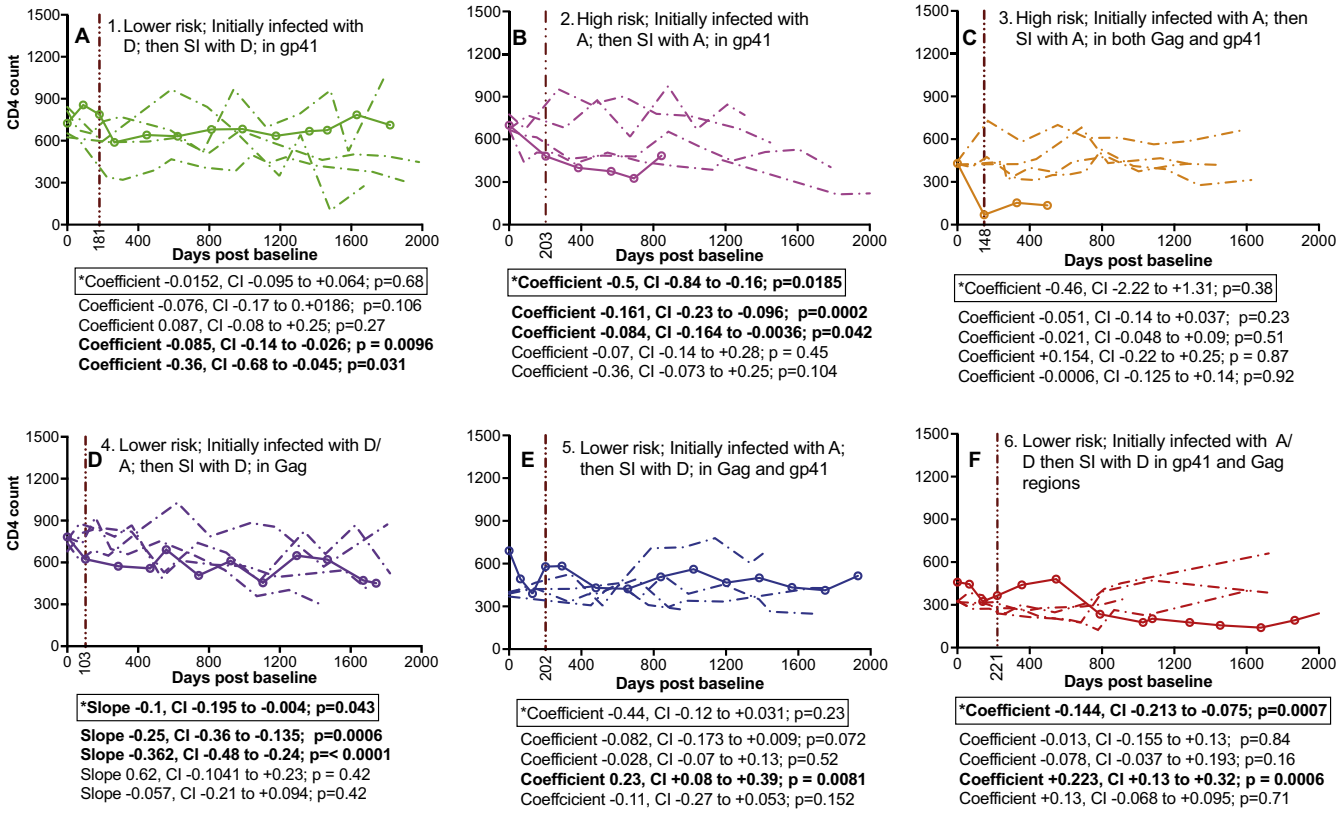
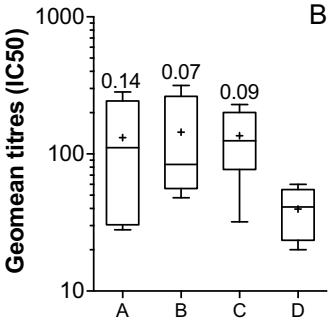


Fig. 1. Participant CD4 T-cell counts over time. This figure illustrates the kinetics of participant CD4 T-cell counts (cells/μl) over time (days). Each graph represents a superinfected case (solid line) and four corresponding singly infected individuals matched by pre-superinfection CD4 T-cell counts (cells/μl) and HIV-1 risky behavior profile (broken lines). Each superinfection case and corresponding singly infected individuals are color-coded and will maintain the same color throughout this manuscript. Regression analyses for CD4 means are computed as coefficients and associated confidence intervals (CI), to determine the trends/slopes over time. Coefficients are given below each graph for a superinfected case (boxed text) and four corresponding singly infected individuals. Positive coefficients indicate increasing trends, and negative coefficients indicate decreasing trends over time. P value ≤0.05 indicate that the trends are statistically significant, and are displayed in bold. Categorization of HIV-1 risky behavior profiles, infecting virus clades, and the estimated timing of superinfection (vertical broken lines) are indicated within each graph. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A

Participant ID	Clade A			Clade B			Clade C			Clade D			% of viruses neutralised
	Q23.17	Q769.d22	Q842.d12	TRO.11	6535.3	RHPA	ConC	CAP45.G3	DU156.12	QA013.H1	QB857.B3	QD835.B5	
1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	20	20	55	405	74	20	102	52	343	20	20	20	**50%**
3	632	183	197	149	87	20	344	82	180	45	20	74	**75%**
4	306	40	690	314	424	70	854	20	113	20	314	20	**67%**
5	47	38	20	44	44	58	84	20	20	20	47	20	17%
6	1472	20	47	585	439	123	1890	68	94	20	157	70	**75%**

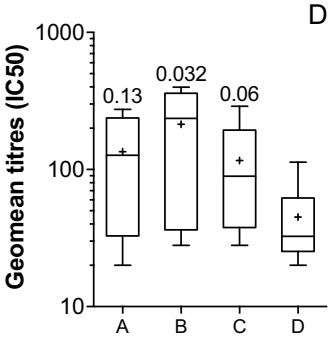
B

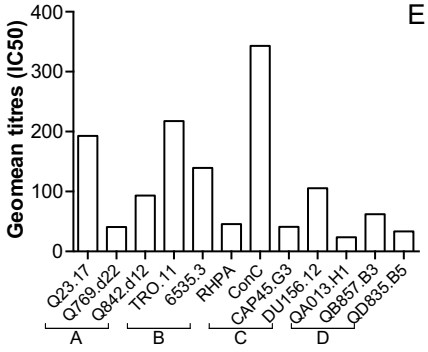


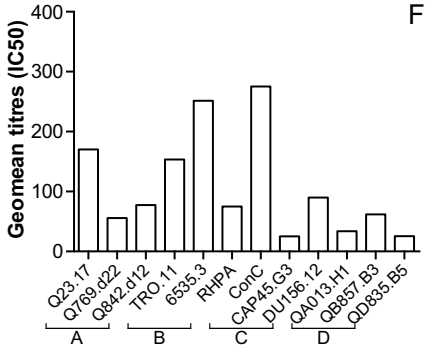
C

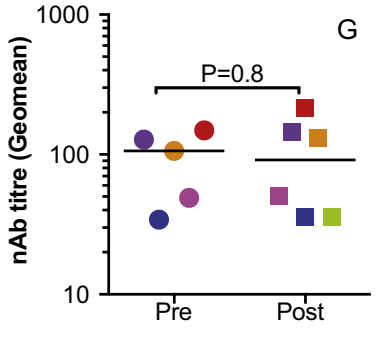
Participant ID	Clade A			Clade B			Clade C			Clade D			% of viruses Neutralised
	Q23.17	Q769.d22	Q842.d12	TRO.11	6535.3	RHPA	ConC	CAP45.G3	DU156.12	QA013.H1	QB857.B3	QD835.B5	
1	47	98	49	20	20	54	52	20	20	46	46	20	25%
2	20	20	20	138	6134	20	104	20	125	20	20	20	33%
3	1062	74	145	582	273	396	760	20	100	66	20	20	**75%**
4	322	96	669	267	387	96	866	20	247	20	229	20	**75%**
5	48	54	20	61	20	50	53	20	63	20	47	20	42%
6	1585	40	113	496	973	87	2307	77	136	60	286	84	**92%**

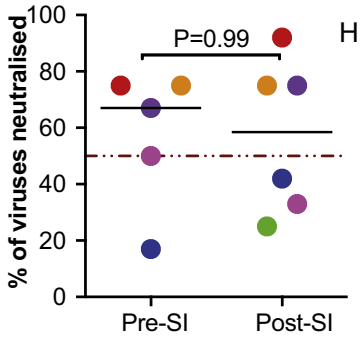
D











I

VIRUSES	1	3	4	5	6
Du156_WT	624	238	218		220
Du156 N332A	358	264	121		40
TRO.11_WT	281	1513	304	54	356
TRO.11_N160K	299	3274	994	65	695
TRO.11_N332A	280	90	605	83	40
Q23.17_WT	330	1311	738		2005
Q23N160K	354	2449	1724		3650
Q23N332A	405	1512	2619		2329
	FOLD EFFECT				
Du156 N332A	1.7	0.9	1.8		5.5
TRON160K	0.9	0.5	0.3	0.7	0.5
TRON332A	1.0	16.8	0.5	0.8	8.9
Q23N160K	0.9	0.5	0.4		0.5
Q23N332A	0.8	0.9	0.3		0.9

Fig. 2. Neutralizing antibody responses before and after superinfection. Quarterly plasmas collected pre- and post-superinfection, were assessed for neutralizing antibody activity against a 12-virus panel comprising clade A (Q23.17, Q769.D22 and Q842.d12), clade B (TRO.11, 6535.3 and RHPA), clade C (ConC, CAP45.G3 and Du156.12) and clade D (QA013.H1, QB57.B3 and QD835.B5). Pre-superinfection neutralization titers (IC50) for virus-plasma pairs are indicated in a heat map as reciprocals of plasma dilution(s) that inhibit 50% of the pseudovirus replication, (A) For ease of analysis, titers below the assay minimum detection limit of 40 were given a value of 20. Geometric mean neutralizing antibody titers of clade D are compared against each of clade A; B; and C pseudoviruses using the *t*-test (B). Post-superinfection neutralizing antibody titers are illustrated in a heat map, (C); geometric means of clade D titres are compared to those of each of the three other clades A, B, C using *t*-tests (D). Geometric means for each virus are illustrated at the pre- (E) and post superinfection (F) time points; and then compared using *t*-test across the two time points (G). Broad neutralization (neutralization of ≥50% viruses) is also compared using Wilcoxon rank sum tests across two time points (H). Each symbol in figures G and H represent an individual superinfected case; the same color code is maintained for each case throughout this manuscript. P values of ≤0.05 are considered statistically significant. Exploratory mapping of some evaluated viruses is summarized in figure I. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Change in Log10 of anti-HIV-1 neutralizing antibodies from superinfection at 12, 24, 36 or 48 visit weeks (350 days) and association with superinfection.

Factor	Univariable model RR (95% CI)	P[†]	Multivariable model RR (95% CI)	P[‡]
Superinfection	0.09 (−0.22, 0.40)	0.6	0.07 (−0.21, 0.35)	0.6
Time dependent CD4 Per 100 cell increase	−0.09 (−0.13, −0.05)	<0.001	−0.09 (−0.13, −0.05)	<0.001
Virus clade				
Clade A	1	<0.001	1	<0.001
Clade B	−0.02 (−0.16, 0.13)		−0.02 (−0.16, 0.13)	
Clade C	0.05 (−0.10, 0.19)		0.05 (−0.09, 0.19)	
Clade D	−0.30 (−0.46, −0.14)		−0.30 (−0.47, −0.14)	

[†] Adjusted for baseline (log) titres.
[‡] Adjusted for baseline (log) titres, time dependent CD4, pseudovirus clade.

361) and C (90, 38–194), p = 0.08, one-way ANOVA, Fig. 2D. Overall, neutralization was largely attributed to the greater sensitivity of Q23.17 (clade A), TRO.11 (clade B), and ConC (clade C) viruses established to be tier 2 [27–29], as well as 6535.3 (clade B) known to be tier 1B [27], Fig. 2E and F. There was no significant difference in neutralizing antibody titres (Fig. 2G) or breadths (Fig. 2H) before and after superinfection, p > 0.05. Exploratory analyses of the antibody specificities revealed that sensitivity of TR011 and Du156 virus strains was due to specific targeting of the Asn332 glycan-dependent residue of the HIV gp120 envelope protein. Use of Du156 and TR0.11 viruses with mutation N332A at the targeted glycan-dependent epitope led to >5.5-fold and 9-fold reduction HIV-1 sensitivity respectively, Fig. 2I. Taken together, these data demonstrate that HIV-1 superinfection can occur despite the presence of established HIV-specific broadly neutralizing antibodies.

3.3. Subsequent broadening of neutralizing antibody responses was only seen when prior antibody levels were narrow

We observed that ensuing broadening of antibody responses varied with the neutralization status prior to superinfection (Fig. 3). For example, only one superinfected individual exhibited significant subsequent neutralizing antibody broadening after superinfection. In this case, neutralizing antibody responses were lacking at baseline; progressively developed after superinfection and significantly broadened with time, (Regression Coefficient + 5 .42; confidence interval 1.54–9.3), p = 0.012, Fig. 3A. On the other hand, in five subjects that got superinfected in the presence of broadly neutralizing antibodies, antibody levels did not significantly change over time (Regression Coefficient p value >0.05) suggesting that their neutralizing antibody response had already plateaued by the time they got superinfected, (Fig. 3B–F). Taken together, these data suggest that boosting of subsequent neutralizing antibodies after superinfection might depend on the prior status of neutralizing antibody development.

3.4. Broad neutralization also occurred in HIV-1 high-risk female sex workers that lacked superinfection

We finally explored whether HIV-1 singly infected individuals possessed neutralizing antibody profiles that were distinct from those seen in the superinfected. We found that baseline neutralization breadth was present in 37% (7/19) of singly infected volunteers; and was more attributable to the greater sensitivity of clade B (30 of 57) and C viruses (31/57) than A (20/57) and D (14/57); p = 0.014, respectively, Fisher's Exact test, (Fig. 4A). As noted before, clade D had lower titers [31, (21–41)] than B [58, (34–81); p = 0.002] and C [67, (35–100); p = 0.016, paired *t*-test, (Fig. 4B). Proportions of singly infected individuals with broad neutralization at baseline (7/19) and at 350 days post-superinfection (13/24) did not significantly differ, p = 0.36, Fig. 4C. Also, antibody titers were not significantly different across clades, (Fig. 4E). In singly infected subjects, antibody levels remained generally leveled (P values for Regression Coefficients >0.5), suggesting that pre-existing levels of neutralizing antibodies were already plateaued; supplementary Fig. 1. Increasing antibody responses as determined by regression coefficients were only seen in two of 24 cases: figure A2, +21.4, p = 0.44 and figure B3: +0.744; p = 0.038), supplementary Fig. 1. Collectively, these data suggest that in HIV-1 chronically individuals where antibody levels are largely plateaued, profiles of neutralizing antibody responses in superinfected subjects may not distinctly differ from those in their singly infected counterparts.

4. Discussion

We evaluated the status of virus-specific neutralizing antibodies in drug-naïve, HIV-1 high-risk behavior, already infected female sex workers to determine if further infection with new HIV-1 strains (superinfection) occurred because of distinctively inferior neutralizing antibody profiles. We found that superinfection occurred in the presence, as well as in the absence of broadly neutralizing antibodies. The later outcome was linked to lack of prior development of neutralizing antibodies. Where superinfection occurred amidst broad neutralization, prior neutralizing antibody responses had already reached their peak. Levels of HIV-specific neutralizing antibodies negatively correlated with concurrent CD4 + T cell counts. Broadening of responses occurred within 9 months of superinfection; but only in one individual that lacked prior antibodies. When superinfection occurred amidst pre-existing neutralization breadth, no further broadening ensued. Clade associations occurred; clade D viruses were the least sensitive, and the non-endemic clade B and C were more sensitive than the locally circulating clades A and D viruses.

Reinfection of infected individuals with additional HIV-1 strains has been most reported in early infection where prior neutralizing antibody responses are low, [1,2,30]. However, this has not always been the case; some superinfection cases have also been detected in the presence of pre-established virus-specific immune responses [3,4]. Such conflicting outcomes are partly attributed to intrinsic differences in study populations, variations in assessed viruses, and differing criteria for matching cases and controls. Although preceding antibodies protected in humans [13], mice [31], and primate models of HIV-1 infection [32], pre-existing, broadly neutralizing antibodies did not prevent superinfection in our cohort. Numerous factors might explain failure of prior antibodies to protect. Antibody breadths and titres did not significantly change from superinfection till 350 days after. This contrasted with others that showed subsequent broadening of antibody responses following superinfection [33,34]. Perhaps neutralizing antibody development in our cohort had peaked. Others showed that anti-HIV-1 neutralizing antibodies develop within two years of infection, peak by four

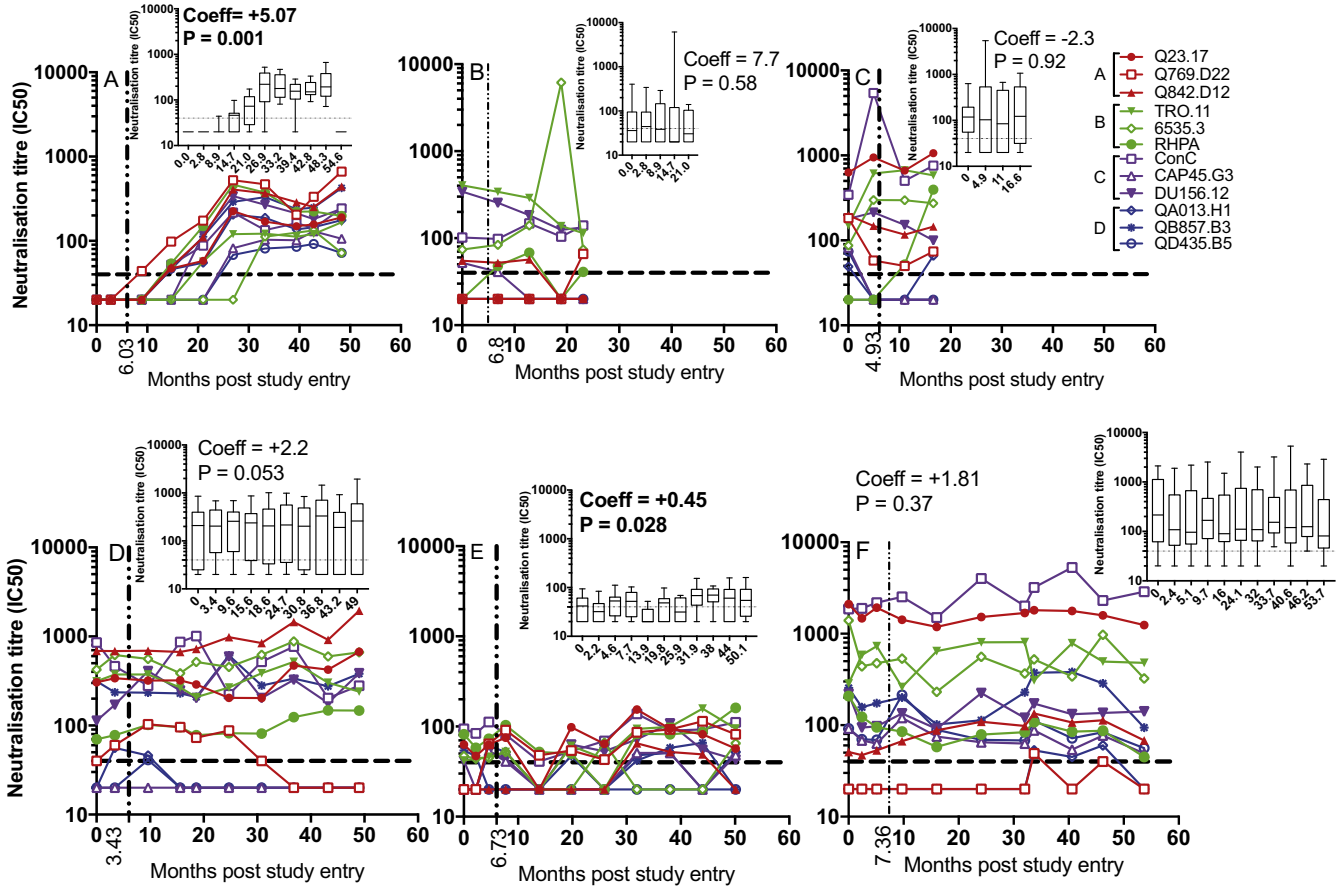


Fig. 3. Kinetics of neutralizing antibody development in HIV-1 high risk, superinfected female sex workers. Retrospective longitudinal stored plasma specimens previously collected from superinfected HIV-1 high risk female sex workers were screened for ability to neutralize clade A, B, C and D pseudoviruses (3 viruses per clade). Each graph (with its inset) represents the longitudinal neutralization of twelve viruses by one superinfected individual. Neutralization is plotted as titres (50% inhibitory titre [IC_{50}]), and summarized as geometric means per time point (insets). Horizontal dotted lines represent cut offs for neutralization sensitivity. Vertical dotted lines represent the estimated superinfection time point (months). Regression analysis was computed for neutralizing antibody means (across virus types) against time to determine the trends. Positive coefficients indicate a positive trend/slope over time, negative coefficients indicate a negative trend. P values $\leq$0.05 indicate that the trend is significant; statistically significant trends are highlighted in bold.

years, with no further increases thereafter [35–40]. We found broad neutralization to be associated with low CD4 T Cells. While this is in line with known associations of neutralization with progressed HIV-1 disease [35,39,41], it also suggests that prior antibodies were not protective. Perhaps the pre-existing antibodies signified likely antigenic stimulation rather than immune protection.

In this cohort, HIV-1 superinfection and primary infection rates were similar [20]. This also suggests that prior neutralizing antibody responses were ineffective at preventing superinfection. Participants CD4 T Cells were significantly declining in the face of neutralizing antibodies further suggesting that concomitant antibodies were ineffective. Immune conditions that can influence HIV-1 infection differ between HIV-1-infected and uninfected individuals. Among the uninfected, prior virus-specific responses are lacking rendering the individuals more vulnerable to infection. In the infected, pre-existing virus-specific antibody responses are typically present, but coexisting confounding factors might influence their protectiveness. The plateaued nature of prior antibodies suggests that infection in this cohort was chronic. Participants had not initiated treatment; persistent viral replication and progressive CD4 cell loss typical of untreated chronic HIV-1 infection might have further compromised their immunity. Data on opportunistic infection episodes was not collected. Future analyses of drug-naïve individuals will need to correct for confounding effects of opportunistic infections on protection from superinfection.

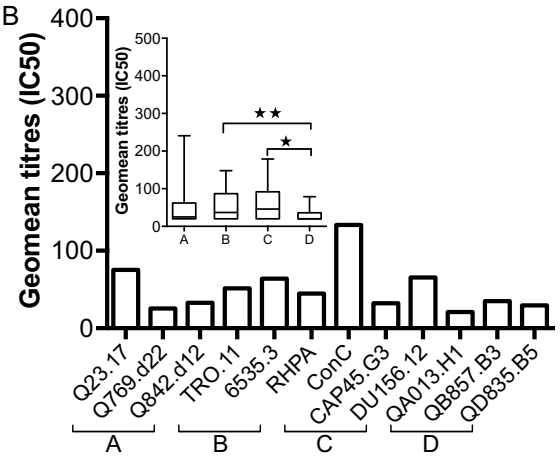
Clade differences in neutralization sensitivities could not be explained under the scope of this study. Others linked this to differential targeting of key epitopes of broad vulnerability by clade A and D [38,42], and to the greater sensitivity clade C viruses [36]. Exploratory analyses showed that clade B and C neutralization breadth specificities were due to the N332 glycan supersite targeting [36,43,44]. However, specimen limitations prevented similar assessments on clades A and D-directed specificities. The significantly lower sensitivity of clade D viruses was in line with established associations of clades with broadly neutralizing antibody responses [35,42,45]; and with the known associations of clade D with rapid HIV-1 disease progression [46–48].

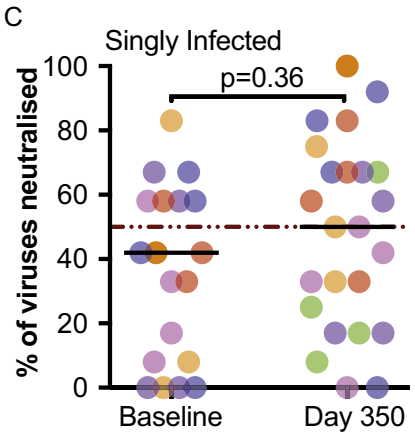
Overall, our data suggests that higher titres and a broader spectrum of neutralizing antibodies are needed to prevent superinfection than was detected here. Tremendous evidence from various studies has shown that broadly neutralizing antibodies given before mucosal exposure can prevent infection in humanized mice and macaques. However, sustaining that protection requires continuous boosting with titres well above those normally found in natural infection [9,49–53].

Perhaps co-circulating viruses had escaped from the co-existing neutralizing antibodies. One caveat to this data is that only heterologous neutralizing antibodies could be assessed. Stored specimens were depleted, isolation of transmitted superinfecting viruses, and evaluation of autologous neutralizing antibodies that might better determine the correlates of protection from superinfection was not

A

	Clade A			Clade B			Clade C			CladeD			% of viruses neutralised
	Q23.17	Q769.d22	Q842.d12	TRO.11	6535.3	RHPA	ConC	CAP45.G3	DU156.12	QA013.H1	QB857.B3	QD835.B5	
2a	20	20	20	20	49	50	121	20	41	20	20	20	17%
2b	128	20	20	55	20	255	68	20	20	20	20	20	33%
2c	795	20	20	85	113	69	175	20	109	20	50	20	58%
2d	20	20	20	20	55	20	40	20	20	20	20	20	8%
3a	20	91	20	53	410	20	153	20	1495	20	20	20	42%
3b	20	20	20	20	59	20	20	20	20	20	20	42	8%
3c	223	208	301	97	223	112	138	47	122	20	191	66	83%
3d	20	20	20	20	20	20	20	20	20	20	20	20	0%
4a	20	20	20	20	20	20	20	20	20	20	20	20	0%
4b	99	20	119	212	175	20	81	20	85	20	188	20	58%
4c	20	20	20	20	20	20	20	20	20	20	20	20	0%
4d	67	20	20	99	471	70	192	104	288	41	276	43	67%
5a	20	20	20	20	97	127	140	146	20	20	20	118	42%
5b	20	41	20	20	20	20	20	20	20	20	20	20	0%
5c	462	20	47	112	139	84	698	65	114	20	48	20	58%
5d	281	20	55	471	442	154	1074	63	2394	20	20	20	67%
6a	1006	20	57	20	20	20	813	67	20	20	20	61	42%
6b	88	20	42	530	20	203	1500	53	142	20	101	48	58%
6c	504	20	67	45	20	20	3062	20	81	20	20	46	33%





D

	Clade A			Clade D			Clade C			Clade D			% of viruses neutralised
	Q23.17	Q769.d22	Q842.d12	TRO.11	6535.3	RHPA	ConC	CAP45.G3	DU156.12	QA013.H1	QB857.B3	QD835.B5	
1a	20	20	20	45	20	20	91	20	72	20	20	20	25%
1b	20	122	311	98	63	3049	97	20	85	78	20	20	67%
1c	20	88	20	20	42	20	20	20	20	20	20	20	17%
1d	20	20	20	20	20	20	121	20	20	20	20	20	8%
2a	45	20	20	20	44	41	124	20	20	20	20	20	33%
2b	149	20	20	47	42	283	91	20	20	20	20	20	42%
2c	120	20	68	81	56	20	60	20	57	20	20	20	50%
2d	20	20	20	20	20	20	20	20	20	20	20	20	0%
3a	20	261	20	20	202	20	396	63	2544	20	20	50	50%
3b	20	20	20	48	76	67	86	59	88	49	43	54	75%
3c	163	140	274	147	361	116	137	49	112	72	210	69	100%
3d	49	49	92	20	20	20	20	20	44	20	20	20	33%
4a	20	20	20	20	78	20	20	20	78	20	20	20	17%
4b	88	20	67	192	85	20	83	60	105	20	66	20	67%
4c	20	20	20	20	82	20	56	20	20	20	20	20	17%
4d	20	20	85	50	162	20	142	47	85	20	164	20	58%
5a	145	20	380	89	214	520	304	57	20	20	20	79	67%
5b	20	20	20	20	20	20	20	20	20	20	20	20	0%
5c	514	20	48	128	71	61	604	89	141	43	20	53	83%
5d	266	20	209	264	488	305	885	135	819	104	106	94	92%
6a	948	20	196	20	62	20	4684	101	178	20	46	90	67%
6b	2424	67	179	115	20	58	13590	4196	346	20	57	939	83%
6c	616	20	63	47	88	88	6082	20	75	20	20	20	58%
6d	20	20	20	213	80	20	42	20	20	57	20	20	33%

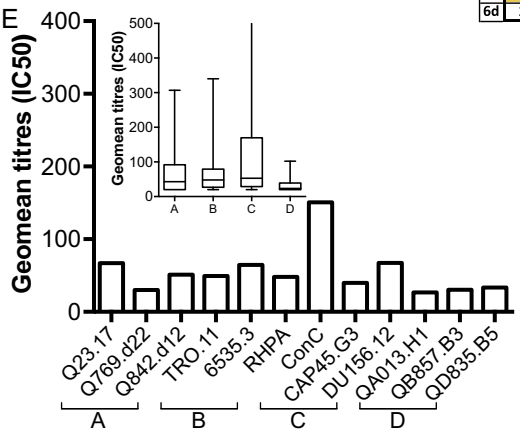


Fig. 4. Broadly neutralizing antibody responses also occurred in singly infected high risk female sex workers. (A) illustrates neutralization titres (IC50) for virus-plasma pairs. Titres are derived as reciprocals of plasma dilution(s) that inhibit 50% of the Pseudovirus infection. All titers below the minimum detection limit of 40 are regarded as 20. Geometric means of baseline neutralizing titers are shown for clade A, B, C and D (B); and then compared for each clade against clade D; $^{*}p < 0.01$ and $^{**}p < 0.001$, (B inset). Only significant differences are indicated. P values ≤ 0.05 are considered significant. Neutralization breadth (neutralization of $\geq 50\%$ viruses) is compared between baseline and day 350. Each symbol represents one singly infected individual. Symbols are color-coded to match the corresponding superinfected case (C). The heat map shows neutralizing antibody titers against the twelve pseudoviruses in singly infected FSWs. Geometric mean titers are also shown 350 days after the baseline time point, figure E. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

possible. However, subtype C studies have linked superinfection with low prior autologous antibodies [1]. A prospective clade A and D superinfection cohort is ongoing to enable comprehensive assessments of autologous antibody determinants of protection from superinfection. Another limitation to this data is that specimen limitations prevented assessments of plasma viral loads in

this cohort. Viral load and duration of infection are the key determinants of broadly neutralizing antibody development [35,36,39]. Study designs controlling for viral loads will be needed to improve assessments of associations of SI with anti-HIV-1 neutralizing antibodies.

Despite the small sample size evaluated, we established that pre-existing broadly neutralizing antibodies can fail to prevent HIV-1 superinfection during chronic infection; and that insufficient neutralization breadth during early antibody development may also fail to prevent superinfection. These data imply that successful antibody-based vaccine strategies to prevent HIV-1 infection might need to deliver much greater breadths of HIV-1 neutralizing antibodies than those normally detected during natural infection.

Disclaimer

This manuscript has not been published in its current form or in a substantially similar form. There are no financial, consultant, institutional and other relationships that might lead to bias or a conflict of interest.

Acknowledgements

This research was jointly funded by the UK Medical Research Council (MRC), and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement. Maintaining of the Good Health for Women Project (GHWP) clinic was co-funded by EDCTP. We thank all the study participants and the dedicated clinical and field staff of the Good Health for Women Project (GHWP) clinic in Kampala. Pseudovirus reagents were obtained through the NIH AIDS Research and Reference reagent program, Division of AIDS, NIAID, NIH; and from collaborations with Prof Lynn Morris' AIDS unit lab at the National Institute of Communicable diseases, Johannesburg, South Africa.

All authors made substantial contributions as follows: (i) conception and design of the study (JS, DS, PK, JL, LM, SN); (ii) Performing single genome sequencing and analyses for detection of superinfection (AR, TQ, SFP, DS); (iii) Specimen and laboratory data acquisition (RN, TH, MM, JS, YM, SN, DS); (iv) Data analysis and interpretation (JS, PK, DS, RN, SN, SK-M, PI, LM), (v) drafting, revising and critical review of manuscript for important intellectual content (JS, DS, PK, LM, TH, AR, TQ), (vi) All authors read and approved the final manuscript; (vii)final approval of the version to be submitted (JS, DS,LM, PK).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2017.11.075.

References

[1] Basu D, Kraft CS, Murphy MK, Campbell PJ, Yu T, Hraber PT, et al. HIV-1 subtype C superinfected individuals mount low autologous neutralizing antibody responses prior to intrasubtype superinfection. Retrovirology 2012;9:76.
[2] Smith DM, Strain MC, Frost SD, Pillai SK, Wong JK, Wrin T, et al. Lack of neutralizing antibody response to HIV-1 predisposes to superinfection. Virology 2006;355:1–5.
[3] Blish CA, Dogan OC, Derby NR, Nguyen MA, Chohan B, Richardson BA, et al. Human immunodeficiency virus type 1 superinfection occurs despite relatively robust neutralizing antibody responses. J Virol 2008;82:12094–103.
[4] Piantadosi A, Chohan B, Chohan V, McClelland RS, Overbaugh J. Chronic HIV-1 infection frequently fails to protect against superinfection. PLoS Pathogens 2007;3:e177.
[5] Walker LM, Phogat SK, Chan-Hui PY, Wagner D, Phung P, Goss JL, et al. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science 2009;326:285–9.
[6] Scheid JF, Mouquet H, Feldhahn N, Seaman MS, Velinzon K, Pietzsch J, et al. Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. Nature 2009;458:636–40.
[7] Doria-Rose NA, Schramm CA, Gorman J, Moore PL, Bhiman JN, DeKosky BJ, et al. Developmental pathway for potent V1V2-directed HIV-neutralizing antibodies. Nature 2014;509:55–62.
[8] Sok D, van Gils MJ, Pauthner M, Julien JP, Saye-Francisco KL, Hsueh J, et al. Recombinant HIV envelope trimer selects for quaternary-dependent antibodies targeting the trimer apex. Proc Nat Acad Sci USA 2014;111:17624–9.
[9] Hessell AJ, Rakasz EG, Poignard P, Hangartner L, Landucci G, Forthal DN, et al. Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. PLoS Pathogens 2009;5:e1000433.
[10] Balazs AB, Chen J, Hong CM, Rao DS, Yang L, Baltimore D. Antibody-based protection against HIV infection by vectored immunoprophylaxis. Nature 2012;481:81–4.
[11] Moldt B, Rakasz EG, Schultz N, Chan-Hui PY, Swiderek K, Weisgrau KL, et al. Highly potent HIV-specific antibody neutralization in vitro translates into effective protection against mucosal SHIV challenge in vivo. Proc Nat Acad Sci USA 2012;109:18921–5.
[12] Saunders KO, Pegu A, Georgiev IS, Zeng M, Joyce MG, Yang ZY, et al. Sustained delivery of a broadly neutralizing antibody in nonhuman primates confers long-term protection against simian/human immunodeficiency virus infection. J Virol 2015;89:5895–903.
[13] Caskey M, Klein F, Lorenzi JC, Seaman MS, West Jr AP, Buckley N, et al. Viraemia suppressed in HIV-1-infected humans by broadly neutralizing antibody 3BNC117. Nature 2015;522:487–91.
[14] Mehandru S, Vcelar B, Wrin T, Stiegler G, Joos B, Mohri H, et al. Adjunctive passive immunotherapy in human immunodeficiency virus type 1-infected individuals treated with antiviral therapy during acute and early infection. J Virol 2007;81:11016–31.
[15] Trkola A, Kuster H, Rusert P, Joos B, Fischer M, Leemann C, et al. Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. Nat Med 2005;11:615–22.
[16] Barouch DH, Whitney JB, Moldt B, Klein F, Oliveira TY, Liu J, et al. Therapeutic efficacy of potent neutralizing HIV-1-specific monoclonal antibodies in SHIV-infected rhesus monkeys. Nature 2013;503:224–8.
[17] Klein F, Halper-Stromberg A, Horwitz JA, Gruell H, Scheid JF, Bournazos S, et al. HIV therapy by a combination of broadly neutralizing antibodies in humanized mice. Nature 2012;492:118–22.
[18] Shingai M, Nishimura Y, Klein F, Mouquet H, Donau OK, Plishka R, et al. Antibody-mediated immunotherapy of macaques chronically infected with SHIV suppresses viraemia. Nature 2013;503:277–80.
[19] Ronen K, McCoy CO, Matsen FA, Boyd DF, Emery S, Odem-Davis K, et al. HIV-1 superinfection occurs less frequently than initial infection in a cohort of high-risk Kenyan women. PLoS Pathogens 2013;9:e1003593.
[20] Redd AD, Ssemwanga D, Vandepitte J, Wendel SK, Ndembi N, Bukenya J, et al. Rates of HIV-1 superinfection and primary HIV-1 infection are similar in female sex workers in Uganda. Aids 2014;28:2147–52.
[21] Vandepitte J, Bukenya J, Weiss HA, Nakubulwa S, Francis SC, Hughes P, et al. HIV and other sexually transmitted infections in a cohort of women involved in high-risk sexual behavior in Kampala, Uganda. Sex Transm Dis 2011;38:316–23.
[22] Vandepitte J, Weiss HA, Bukenya J, Nakubulwa S, Mayanja Y, Matovu G, et al. Alcohol use, mycoplasma genitalium, and other STIs associated With HIV incidence among women at high risk in Kampala, Uganda. J Acq Immune Def Synd 2013;62:119–26.
[23] Redd AD, Quinn TC, Tobian AA. Frequency and implications of HIV superinfection. Lancet Infect Dis 2013;13:622–8.
[24] Moore PL, Ranchobe N, Lambson BE, Gray ES, Cave E, Abrahams MR, et al. Limited neutralizing antibody specificities drive neutralization escape in early HIV-1 subtype C infection. PLoS Pathogens 2009;5:e1000598.
[25] Montefiori DC. Measuring HIV neutralization in a luciferase reporter gene assay. Methods Mol Biol 2009;485:395–405.
[26] Sarzotti-Kelsoe M, Bailer RT, Turk E, Lin CL, Bilska M, Greene KM, et al. Optimization and validation of the TZM-bl assay for standardized assessments of neutralizing antibodies against HIV-1. J Immunol Methods 2014;409:131–46.
[27] Li M, Gao F, Mascola JR, Stamatatos L, Polonis VR, Koutsoukos M, et al. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J Virol 2005;79:10108–25.
[28] Blish CA, Nedellec R, Mandaliya K, Mosier DE, Overbaugh J. HIV-1 subtype A envelope variants from early in infection have variable sensitivity to neutralization and to inhibitors of viral entry. Aids 2007;21:693–702.
[29] Kothe DL, Li Y, Decker JM, Bibollet-Ruche F, Zammit KP, Salazar MG, et al. Ancestral and consensus envelope immunogens for HIV-1 subtype C. Virology 2006;352:438–49.
[30] Wagner GA, Landais E, Caballero G, Phung P, Kosakovsky Pond SL, Poignard P, et al. Intrasubtype B HIV-1 superinfection correlates with delayed neutralizing antibody response. J Virol 2017.
[31] Church JA. Vectored Immunoprophylaxis Protects Humanized Mice From Mucosal HIV Transmission. Pediatrics 2014;134(Suppl 3):S184–5.
[32] Saunders KO, Wang L, Joyce MG, Yang ZY, Balazs AB, Cheng C, et al. Broadly neutralizing human immunodeficiency virus type 1 antibody gene transfer

protects non-human primates from mucosal simian-human immunodeficiency virus infection. J Virol 2015.
[33] Cortez V, Odem-Davis K, McClelland RS, Jaoko W, Overbaugh J. HIV-1 superinfection in women broadens and strengthens the neutralizing antibody response. PLoS Pathogens 2012;8:e1002611.
[34] Powell RL, Kinge T, Nyambi PN. Infection by discordant strains of HIV-1 markedly enhances the neutralizing antibody response against heterologous virus. J Virol 2010;84:9415–26.
[35] Gray ES, Madiga MC, Hermanus T, Moore PL, Wibmer CK, Tumba NL, et al. The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection. J Virol 2011;85:4828–40.
[36] Landais E, Huang X, Havenar-Daughton C, Murrell B, Price MA, Wickramasinghe L, et al. Broadly Neutralizing Antibody Responses in a large longitudinal sub-saharan HIV primary infection cohort. PLoS Pathogens 2016;12:e1005369.
[37] Mikell I, Sather DN, Kalams SA, Altfeld M, Alter G, Stamatatos L. Characteristics of the earliest cross-neutralizing antibody response to HIV-1. PLoS Pathogens 2011;7:e1001251.
[38] Kwong PD, Mascola JR, Nabel GJ. Broadly neutralizing antibodies and the search for an HIV-1 vaccine: the end of the beginning. Nat Rev Immunol 2013;13:693–701.
[39] Doria-Rose NA, Klein RM, Daniels MG, O'Dell S, Nason M, Lapedes A, et al. Breadth of human immunodeficiency virus-specific neutralizing activity in sera: clustering analysis and association with clinical variables. J Virol 2010;84:1631–6.
[40] Sather DN, Armann J, Ching LK, Mavrantoni A, Sellhorn G, Caldwell Z, et al. Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. J Virol 2009;83:757–69.
[41] Euler Z, van Gils MJ, Bunnik EM, Phung P, Schweighardt B, Wrin T, et al. Cross-reactive neutralizing humoral immunity does not protect from HIV type 1 disease progression. J Infect Dis 2010;201:1045–53.
[42] Dreja H, O'Sullivan E, Pade C, Greene KM, Gao H, Aubin K, et al. Neutralization activity in a geographically diverse East London cohort of human immunodeficiency virus type 1-infected patients: clade C infection results in a stronger and broader humoral immune response than clade B infection. J General Virol 2010;91:2794–803.
[43] Simonich CA, Williams KL, Verkerke HP, Williams JA, Nduati R, Lee KK, et al. HIV-1 neutralizing antibodies with limited hypermutation from an infant. Cell 2016;166:77–87.
[44] Bhiman JN, Anthony C, Doria-Rose NA, Karimanzira O, Schramm CA, Khoza T, et al. Viral variants that initiate and drive maturation of V1V2-directed HIV-1 broadly neutralizing antibodies. Nat Med 2015;21:1332–6.
[45] Bandawe GP, Moore PL, Werner L, Gray ES, Sheward DJ, Madiga M, et al. Differences in HIV type 1 neutralization breadth in 2 geographically distinct cohorts in Africa. J Infect Dis 2015;211:1461–6.
[46] Kaleebu P, French N, Mahe C, Yirrell D, Watera C, Lyagoba F, et al. Effect of human immunodeficiency virus (HIV) type 1 envelope subtypes A and D on disease progression in a large cohort of HIV-1-positive persons in Uganda. J Infect Dis 2002;185:1244–50.
[47] Kiwanuka N, Laeyendecker O, Robb M, Kigozi G, Arroyo M, McCutchan F, et al. Effect of human immunodeficiency virus type 1 (HIV-1) subtype on disease progression in persons from Rakai, Uganda, with incident HIV-1 infection. J Infect Dis 2008;197(5):707–13.
[48] McKinnon LR, Nagelkerke NJ, Kaul R, Shaw SY, Capina R, Luo M, et al. HIV-1 clade D is associated with increased rates of CD4 decline in a Kenyan cohort. PLoS One 2012;7:e49797.
[49] Rudicell RS, Kwon YD, Ko SY, Pegu A, Louder MK, Georgiev IS, et al. Enhanced potency of a broadly neutralizing HIV-1 antibody in vitro improves protection against lentiviral infection in vivo. J Virol 2014;88:12669–82.
[50] Balazs AB, Ouyang Y, Hong CM, Chen J, Nguyen SM, Rao DS, et al. Vectored immunoprophylaxis protects humanized mice from mucosal HIV transmission. Nature Med 2014;20:296–300.
[51] Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, et al. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med 2000;6:207–10.
[52] Pegu A, Yang ZY, Boyington JC, Wu L, Ko SY, Schmidt SD, et al. Neutralizing antibodies to HIV-1 envelope protect more effectively in vivo than those to the CD4 receptor. Sci Translat Med 2014;6:243ra88.
[53] Willey R, Nason MC, Nishimura Y, Follmann DA, Martin MA. Neutralizing antibody titers conferring protection to macaques from a simian/human immunodeficiency virus challenge using the TZM-bl assay. AIDS Res Human Retrov 2010;26:89–98.