Comprehensive Characterization of Humoral Correlates of Human Immunodeficiency Virus 1 Superinfection Acquisition in High-risk Kenyan Women

Keshet Ronen a,b,c,1, Adam S. Dingens a,c,d,1, Susan M. Graham b,c,e, Walter Jaoko f, Kishor Mandalia b, R. Scott McClelland b,c,e,g, Julie Overbaugh a,h,*

a Human Biology Division, Fred Hutch Cancer Research Center, 1100 Fairview Ave N., Seattle, WA 98109, USA
b Department of Global Health, University of Washington, 1959 NE Pacific Street, Seattle, WA 98195, USA
c Department of Epidemiology, University of Washington, 1959 NE Pacific Street, Seattle, WA 98195, USA
d Molecular and Cellular Biology Program, University of Washington, 1959 NE Pacific Street, Seattle, WA 98195, USA
e Department of Medicine, University of Washington, 1959 NE Pacific Street, Seattle, WA 98195, USA
f Department of Medical Microbiology, University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya
g Institute of Tropical and Infectious Disease, University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya
h Public Health Sciences Division, Fred Hutch Cancer Research Center, 1100 Fairview Ave N., Seattle, WA 98109, USA

1 Equal contribution.

1 Corresponding author at: Human Biology Division, Fred Hutch Cancer Research Center, 1100 Fairview Ave N., Seattle, WA 98109, USA.

E-mail address: joverbau@fredhutch.org (J. Overbaugh).

1 Equal contribution.

HIV-1 superinfection, in which an infected individual acquires a second HIV-1 infection from a different partner, is one of the only settings in which HIV acquisition occurs in the context of a pre-existing immune response to natural HIV infection. There is evidence that initial infection provides some protection from superinfection, particularly after 6 months of initial infection, when development of broad immunity occurs. Comparison of the immune response of superinfected individuals at the time of superinfection acquisition to that of individuals who remain singly infected despite continued exposure can shed light on immune correlates of HIV acquisition to inform prophylactic vaccine design. We evaluated a panel of humoral immune responses in the largest published cohort of superinfected individuals (n = 21), compared to a set of 3:1 matched singly infected controls from the same cohort. The immune functions studied included plasma neutralization, plasma and cervical antibody-dependent cellular cytotoxicity, and plasma IgG and IgA binding to a panel of 18 envelope antigens, including correlates of HIV acquisition in the RV144 vaccine trial, IgG binding to V1V2 and IgA binding to gp140. Association between each immune function and HIV superinfection was evaluated using conditional logistic regression. No significant associations were detected between any of the immune functions and superinfection acquisition. This study constitutes the most comprehensive and detailed characterization of multiple immune correlates of superinfection to date. The results suggest that immune responses not commonly measured in current HIV studies may be important in protection from HIV infection, and these or a more robust humoral response than that seen in naturally infected women may be needed for a protective vaccine.

Corresponding author at: Human Biology Division, Fred Hutch Cancer Research Center, 1100 Fairview Ave N., Seattle, WA 98109, USA.

E-mail address: joverbau@fredhutch.org (J. Overbaugh).

1 Equal contribution.
HIV-specific immune response. SI has been documented in a number of settings (Altfeld et al., 2002; Smith et al., 2004; Chohan et al., 2005; Piantadosi et al., 2007; Redd et al., 2012; Ronen et al., 2013). Early epidemiologic studies in several high-risk cohorts suggested the incidence of SI was similar to that of initial infection (Smith et al., 2004; Kraft et al., 2010; Redd et al., 2013). However, analysis of a larger cohort of SI cases (n = 21) from the Mombasa Cohort, a cohort of high-risk women in Mombasa, Kenya, suggests that HIV-infected individuals are partially protected from SI: the incidence of SI was roughly half that of initial infection in the cohort after adjustment for differences in risk behavior, with evidence of greater protection after 6 months of infection, suggesting a potential immune mechanism (Ronen et al., 2013). Comparison of immune responses immediately before SI acquisition with responses among individuals exposed to HIV who do not acquire SI is therefore a promising strategy to identify immune correlates of HIV acquisition. Importantly, these correlates may differ from correlates of virologic control after establishment of chronic infection.

To date, no studies have examined the immune correlates identified in the RV144 trial for their possible role in protection against SI. Moreover, studies that have investigated other humoral correlates of SI, which have had conflicting findings, have generally been limited to examining one response at a time and small numbers of cases. Two case-controls studies in the Mombasa Cohort found no difference between cases and singly-infected controls: one included 6 SI cases and investigated neutralizing antibody breadth and potency (Blish et al., 2008; UNAIDS, 2016) and the other included 12 SI cases and investigated antibody-dependent cellular viral infection (ADCV) (Haynes et al., 2012; Forthal et al., 2013). However, studies in other cohorts, each comparing 3 SI cases to singly-infected controls, have suggested lower autologous neutralizing antibody (Altfeld et al., 2002; Smith et al., 2004; Chohan et al., 2005; Smith et al., 2006; Piantadosi et al., 2007; Basu et al., 2012; Redd et al., 2012; Ronen et al., 2013) and antibody-dependent cellular cytotoxicity (ADCC) (Basu et al., 2014) in SI cases than controls. Given these conflicting findings, larger studies are needed. Additionally, because synergistic or antagonistic relationships likely exist between different immune functions, any correlate of protection may in fact consist of a combination of responses, which would be missed in studies of a single immune measure. Indeed, interaction analysis in the RV144 study showed that in the presence of low levels of Env-specific IgA, ADCC activity and infection risk were inversely associated, while in the presence of high Env-specific IgA, no such association was present (Haynes et al., 2012). This finding has been hypothesized to be due to competition between binding antibodies (Tomaras et al., 2013). Evaluating multiple immune correlates in aggregate may provide a more nuanced understanding of HIV acquisition risk.

Here, we present a comprehensive analysis of an array of humoral immune responses in 21 SI cases from the Mombasa Cohort compared to individually-matched singly infected controls from the same cohort. This study constitutes the most detailed characterization of multiple immune correlates of SI in the largest group of SI cases analyzed to date. To score neutralizing antibody activity, we used a previously published 4-pseudovirus panel (Cortez et al., 2015). This panel was constructed to include diverse envelope variants isolated early in infection whose neutralizing profiles could provide a range of breadth and potency scores (scoring described below). We first identified variants whose IC50 values varied across individuals and were predictive of NAb breadth scores generated using larger pseudovirus panels in previous studies in the Mombasa Cohort (Blish et al., 2008; Piantadosi et al., 2009; Cortez et al., 2012). A subset of 4 viruses from these panels was found to be predictive of findings based on the larger panels: Q461.d1 (Tier 1b, subtype A) (Long et al., 2002), QD435.100M.a4 (Tier 2, subtype D) (Blish et al., 2009), Q842.d16 (Tier 2, subtype A) (Long et al., 2002), Du156.12 (Tier 2, subtype C) (Li et al., 2006). For example, in a prior study of breadth at 5 years post initial infection among singly and superinfected individuals (Cortez et al., 2012), superinfected individuals had on average 1.68 (95% CI 1.24–2.26) times greater breadth scores than singly infected controls when using an 8-virus panel. The smaller 4-virus panel estimated 1.65 (95% CI 1.08–2.50) times greater breadth scores in superinfected than singly infected controls. Pseudoviruses were produced in HEK 293T cells by cotransfecting equimolar concentrations of the cloned env gene and Q3Δenv (Long et al., 2002), a subtype A full-length proviral clone with a partial deletion in env, using Fugene-6. Forty-eight hours post-transfection,
supernatants were harvested, filtered through a 0.22 μm filter and
filtered on TZM-bl cells.

Case-control sets were assessed for neutralizing antibody activity if
specimens were dated at least two months post initial infection,
allowing for development of a detectable neutralizing antibody re-
sponse (n = 13 cases, 39 controls). We confirmed there was measurable
neutralization activity before this cut-off by assessing neu-
tralization activity of 4 representative early cases (<2 months post-in-
fection) and their 12 match controls against a neutralization sensitive
virus (Q461.d1). Of the 16 early samples tested, 15 had undetectable
neutralization activity (IC50 < 100) and one control sample showed
modest neutralization (IC50 241.2) (data not shown). Plasma samples
were assessed for their ability to neutralize the pseudovirus panel
using a TZM-bl neutralization assay as previously described (Blish et
al., 2007). Briefly, five two-fold dilutions of heat-inactivated plasma
(from 1:100 to 1:1600) were incubated in duplicate with 500
pseudovirus infectious particles for one hour before the addition of
10,000 TZM-bl reporter cells in the presence of 10 μg/mL DEAE-dextran.
Forty-eight hours post-infection, infectivity was assessed by β-galacto-
sidase activity using Gal-Screen (Life Technologies). IC50s (reciprocal
plasma dilution at which 50% of viruses are neutralized) were calculated
based on linear interpolation of the percent neutralization curve. The
assay was performed using a Tecan Freedom Evo 150 liquid handling
robot, with cases and control always being run on the same plate. The
assay was run twice; if the two IC50 values were not within 3-fold of
one another for a given sample, the assay was repeated a third time
for that case-control set. The final IC50 value was calculated as the geo-
metric mean of all available replicates. Pooled HIV-positive plasma and
plasma from an HIV-negative individual were used as positive and neg-
ative controls respectively; both were tested against all viruses in each
experiment. To assess background neutralization, plasma samples
were also tested for neutralization of a pseudovirus bearing SIVMneCL8
Env (Pineda et al., 2007).

Plasma neutralization breadth and potency were scored based on
TZM-bl neutralization assays against the panel of pseudoviruses as pre-
viously described (Cortez et al., 2015). Briefly, breadth scores were cal-
culated by adding one point for each virus that a plasma sample
neutralized at an IC50 greater than the median IC50 value for that
virus across all plasma samples. Potency was scored as the sum of the
ratio of the plasma sample’s IC50 to the cohort median IC50 for each
virus. For score calculation purposes, if a plasma sample did not display
>50% neutralization at the lowest dilution tested, it was given an IC50
value of 50, midway between zero and the lower limit of detection
(100). If the IC50 value was greater than the highest dilution tested, it
was given a value of 1600, the upper limit of our assay. In the small
number of cases in which background neutralization of SIVMneCL8
pseudovirus was observed (IC50 > 100, occurring in 7 of 176 plasma-
SIVMneCL8 assays), we assigned that IC50 value as the lower limit of de-
tection for that plasma sample in that experiment. We accordingly ad-
justed the IC50 value assigned to that plasma sample for any virus that
had an IC50 value below this new limit of detection to halfway be-
tween this limit and zero. In addition to calculating breadth and potency
scores, geometric mean IC50 was calculated across the pseudovirus
panel for confirmatory analysis.

2.4. Antibody-dependent Cellular Cytotoxicity (ADCC) Assay

The Rapid Fluorometric ADCC (RF-ADCC) assay was performed as
described in (Gómez-Román et al., 2006; Mabuka et al., 2012). Briefly,
CEMNkr cells (AIDS Research and Reference Reagent Program, MAID,
NIH) were double labeled with PKH26-cell membrane dye (Sigma Al-
drich) and cytoplasmic-staining CFSE dye (Vybrant CFDA SE Cell Tracer
Kit, Life Technologies) and coated with BL035.W6M.Env.C1 gp120 pro-
tein (Immune Tech) for 1 h at room temperature at a ratio of 1.5 μg
protein (1 μg/mL) per 100,000 double-stained target cells. Coated target
cells were washed once with complete RPMI media (Gibco)
supplemented with 10% FBS (Gibco), 4.0 mM Glutamax (Gibco) and
1% antibiotic-antimycotic (Life Technologies). Samples were diluted in
complete RPMI media and mixed with 5 × 10^5 target cells for 10 min at
room temperature. Peripheral blood mononuclear cells (PBMCs)
from an HIV-negative donor were added at a ratio of 50 effector cells:
1 target cell. Coated target cells, antibody dilutions and effector cells
were co-cultured for 4 h at 37 °C before being fixed in 150 μL 1% parafor-
maldehyde (Affymetrix) and acquired by flow cytometry (LSRII, BD).
ADCC activity was defined as the percent of PE-positive, FITC-negative
cells, analyzed using FlowJo software (TreeStar). Plasma samples were
run at a 1:10,000 dilution. Pooled IgG from HIV-positive individuals,
HIVg (NIH AIDS Reagent Program), was used as a positive control and
target cells coated with SIV gp120 were used as a negative control. Cerv-
ical samples were run at a 1:3 or 1:20 dilution, whichever achieved an
IgG concentration of 5000–50,000 ng/mL (the RFADCC assay dynamic
range) based on IgG ELISA data. Positive and negative control samples
in assays of cervical samples contained 10% DMSO to match the cervical
samples. Samples were run in duplicate in each assay. Two biological
replicates of each assay were performed, using PBMCs from two differ-
ent donors. Within each assay, mean ADCC activity against SIV gp120-
coated cells was subtracted from experimental values as background.
Percent ADCC activity was normalized to HIVg-positive control activity
as described previously (Milligan et al., 2015) and the mean of the two
biological replicates calculated.

2.5. Antibody Binding Assays

The Binding Antibody Multiplex Assay (BAMA) was used to measure
IgG and IgA binding to a panel of antigens, as previously described
(Tomaras et al., 2008; Haynes et al., 2012; Fouda et al., 2015). Briefly,
carboxylated fluorescent beads (Luminex) were covalently coupled to
antigens. Coupled beads were incubated with samples diluted in 5% goat
serum (Sigma), 1% NFDM, 0.05% Tween. After a 30-min incubation
at room temperature, the beads were washed in PBS pH 7.4 containing
1% BSA and 0.05% Tween. Biotin-conjugated mouse anti-human-IgG
(Southern Biotech) or goat anti-human-IgA (Jackson Immunoresearch)
were used at 4 μg/mL for detection. After a 30-minute incubation at
room temperature, the beads were washed and incubated with 1:100
Streptavidin-PE (BD Pharmingen) for 30 min at room temperature.
Beads were washed and acquired using a Bio-Plex 200 instrument
(Bio-Rad Laboratories). Results were expressed as mean fluorescence
intensity (MFI).

The antigen panel used consisted of: gp140 consensus protein for
clade A (Duke Human Vaccine Institute Protein Production Facility);
gp120 protein from BG505.W6M.ENV.C1, BL035.W6M.ENV.C1, C2-
94UG114, ZM109F.PB4, SF162 (Immune Tech); v1v2 scaffolds
MulVgp70-caseA2_v1v2 (Immune Tech), 2J9C-ZM35_v1v2, 1FD6-Fc-
ZM109_v1v2 (jiang et al., 2016); v3 consensus peptides for clades A
(CTRPNNTKRSIRPGQAFQYATGDIGDIRQAH), B (CTRPNNNTKRSI-
PGRPAFTYETIIGDIRQAH), C (CTRPNNNTKRSIRPGQFTYATGDIGDIR-
QAH), D (CTRPNTNRTKTPGQATYATGDIGDIRQAH) (Genscript);
scalloped proteins MulV gp70 (Duke Human Vaccine Institute Protein
Production Facility), 2J9C and 1FD6-Fc (jiang et al., 2016); SOSIP Env trimer
BC505 (courtesy of Marit van Gils, Rogier Sanders and John Moore
(Sanders et al., 2013)); gp41 protein from MN (AIDS Reagent Program),
gp41 ectodomain from 2A,1197/M; C1 consensus peptide for clade A
(MHTDJISLWDQSKPCVKLPLC) (JPT Peptide Technologies); resurface
Env core protein (RSC3) and CD4-binding site defective mutant
(RSC3Δ3711) (construct obtained from AIDS Reagent Program and
produced as described in (Cortez et al., 2015)); SIV gp120 (Immune
Tech). gp120 proteins were purified prior to conjugation to beads by
gel filtration using a HiLoad™ 16/60 Superdex 200 column (GE
Healthcare) equilibrated with phosphate-buffered saline (1 × PBS,
ph 7.4, 1 mM EDTA, 0.02% NaN3). Fractions containing monomeric
gp120 were pooled.
Sample dilutions used in the assay were empirically determined for each antigen based on experiments with plasma and cervical samples from 6 randomly selected participants in other Kenya-based cohorts. For each antigen and sample type, the dilution that yielded an MFI in the linear range of the assay (100–50,000 units) was selected. Samples for detection of IgG binding were whole plasma. For detection of IgA binding, plasma was depleted of IgG by processing through a protein G spin plate (Pierce). Most IgA binding was assayed using plasma that had been depleted by passing the sample over the column twice. One replicate of the samples used to measure binding to V1V2 scaffolds, unconjugated scaffolds, gp140 and C1 used samples depleted by passing the sample over the column three times. IgA and IgG ELISAs on a subset of samples suggested depleting twice or three times yielded similar levels of IgG depletion and negligible loss of IgA (data not shown). Samples were run at 1:200 dilution for detection of IgG binding to consensus A gp140, V1V2 scaffolds, scaffold proteins, gp120 BC505.W6M.ENV.C1, gp120 BL035.W6M.Env.C1, gp120 ZM109.FPB4, gp120 SF162, Env trimer, and consensus D V3. Samples were run at 1:800 dilution for detection of IgG binding to RSC3, RSC3 Δ371I, consensus A1 V3, B V3 and D V3, and gp120 C2-94UG114. Samples were run at 1:10,000 for detection of IgG binding to gp41 antigens. Samples were run at 1:10 for detection of IgA binding to all antigens. SIV gp120 coated beads were run as negative controls at each sample dilution. Strong correlation was observed between sample binding to SIV gp120 and mock-conjugated (blank) beads (data not shown). Samples were run in duplicate on each plate and two biological replicates of each assay were performed.

To control for non-specific background binding to beads, a similar approach was used to that by Haynes et al. (Haynes et al., 2012). For each antigen, a negative control was designated: for V1V2 scaffolds MulVgp70-caseA2, v1v2, 2J9C-2M53, v1v2 and 1F6D-Fc-ZM109_v1v2 this was matched scaffold proteins MulV gp70, 2J9C and 1F6D-Fc respectively; for RSC3, RSC3 Δ371I; for all other antigens, SIV gp120. MFIs ≤ 100 units above the appropriate negative control MFI were set to zero. The negative control MFI was subtracted from the remaining MFIs within each biological replicate. The arithmetic mean of control-subtracted MFI of the two biological duplicates was calculated. Mean control-subtracted MFIs were log transformed for statistical analysis.

2.6. Statistical Analysis

Conditional logistic regression was used to evaluate differences in the immune correlates studied in SI cases compared with matched singly infected controls. Continuous predictors were tested in the model unless the proportion of data points below the lower limit of detection exceeded 40%, in which case data were dichotomized as detectable or undetectable. Contemporaneous viral load was identified a priori as a potential confounder based on its reported association with the development of humoral immunity (Piantadosi et al., 2009; Gray et al., 2011; Mabuka et al., 2012) and SI (Ronen et al., 2014). This variable was included as an adjustment in conditional logistic regression models. Statistical analyses were performed using Stata 12 and R. Two-sided Wald tests were used to evaluate statistical significance, at an α level of 0.05.

A statistical analysis plan was specified a priori. Our primary predictors of interest were neutralizing antibody breadth, neutralizing antibody potency, geometric mean neutralizing IC50, ADCC activity, IgG binding to a panel of V1V2 antigens (individually and the arithmetic mean binding to the panel), and IgA binding to gp140. We additionally performed hypothesis-generating analyses exploring the association between SI acquisition and secondary predictors including binding to gp120, V3, C1, gp41, and Env trimer. Only crude models were performed for these exploratory analyses. These results were viewed as exploratory; p-values for these analyses were not adjusted for multiple comparisons.

3. Results

3.1. Characteristics of SI Cases and Singly Infected Controls

Table 1 summarizes the characteristics of the 21 SI case samples and their corresponding 63 singly infected controls, matched to cases based on time since initial infection and initial viral subtype. They are representative of the three main viral subtypes circulating in Kenya (subtypes A, D and C), and include both inter- and intra-subtype superinfections (Table S1). The median time from initial infection to SI acquisition was 391 days (inter-quartile range [IQR] 112–878), where the timing of SI was defined as the midpoint between the last singly infected and first dually infected timepoints. The median timing of the last singly infected sample available was 255 days after initial infection (IQR 58–714). The number of cases included in the different analyses differed based on sample availability. Of the 21 cases, plasma samples immediately pre-SI were available for 20 cases, and cervical swab samples were available for 17 cases. Samples used for neutralization assays were restricted to the 13 whose samples were at least 2 months post-infection, due to the delay in development of detectable neutralization activity. Three matched controls were selected for each case. With the exception of the samples used for neutralization, which were later in infection by design, the subset of cases with available samples had similar timing, contemporaneous VL and sexual behavior characteristics to the full set of 21 cases. Cases and controls had similar sexual behavior characteristics.

3.2. No Association Between SI Acquisition and Plasma or Cervical Total IgG Titer

Fig. S1 summarizes total IgG titer in plasma and cervical samples from SI cases and controls. In both compartments, no association was found between total IgG titer and SI acquisition by conditional logistic regression: the odds ratio (OR) was 0.98 (95% confidence interval [CI]

### Table 1

| Characteristics of cases and controls. | Cases | Controls |
|----------------------------------------|------|---------|
| | Median | IQR | Median | IQR |
| Full set | n = 21 | | | |
| Estimated timing of SI (dpi) | 391 | 112–878 | | |
| Last singly infected timepoint | 255 | 58–714 | | |
| Contemporaneous plasma VL (log copies/mL) | 4.51 | 3.97–5.21 | | |
| Sex partners in the last week | 1 | 0–1 | 0 | 0–0 |
| Unprotected sex acts in the last week | 0 | 0–0 | | |
| Plasma assayed for binding & ADCC | n = 20 | | | |
| Estimated timing of SI (dpi) | 420 | 110–881 | | |
| Assayed timepoint (dpi) | 260 | 57–723 | 251 | 56–724 |
| Contemporaneous plasma VL (log copies/mL) | 4.52 | 4.07–5.21 | 4.91 | 4.36–5.51 |
| Sex partners in the last week | 1 | 0–1 | 1 | 0–1 |
| Unprotected sex acts in the last week | 0 | 0–0 | 0 | 0.0–0.5 |
| Plasma assayed for neutralization | n = 13 | | | |
| Estimated timing of SI (dpi) | 861 | 391–1041 | | |
| Assayed timepoint (dpi) | 341 | 264–996 | 350 | 254–997 |
| Contemporaneous plasma VL (log copies/mL) | 4.30 | 4.07–4.94 | 4.76 | 4.30–5.35 |
| Sex partners in the last week | 1 | 0–0.33 | 0.67 | 0.33–1.00 |
| Unprotected sex acts in the last week | 0 | 0–0 | 0.00–0.33 | |
| Cervical swabs assayed for ADCC | n = 17 | | | |
| Estimated timing of SI (dpi) | 448 | 293–890 | | |
| Assayed timepoint (dpi) | 275 | 155–749 | 271 | 152–750 |
| Contemporaneous plasma VL (log copies/mL) | 4.40 | 3.97–5.16 | 4.76 | 4.29–5.29 |
| Sex partners in the last week | 1 | 0–1 | 0.5 | 0.0–1.0 |
| Unprotected sex acts in the last week | 0 | 0–0 | 0 | 0–0 |

Controls matched to cases on time since HIV infection and initial viral subtype. ADCC = antibody-dependent cell-mediated cytotoxicity, Dpi = days post-infection, IQR = interquartile range, SI = superinfection, VL = viral load.
3.3. No Association Between SI Acquisition and Neutralizing Antibody Breadth and Potency

Fig. 2 summarizes neutralizing antibody breadth and potency in SI cases and controls, calculated based on plasma neutralization against a panel of 4 viruses selected to be representative of results of larger virus panels based on prior studies (as described in Materials and Methods). In conditional logistic regression, no association was found between either breadth or potency and SI acquisition. For antibody breadth a crude OR of 1.00 was estimated (95% CI 0.52–1.93, p = 1.00). Adjustment for contemporaneous VL did not alter the point estimate appreciably (adjusted OR [aOR] 1.35, 95% CI 0.73–2.53, p = 0.33). Similarly, for neutralizing potency, a crude OR of 0.93 was estimated (95% CI 0.75–1.15, p = 0.51), with a VL-adjusted OR of 0.99 (95% CI 0.74–1.32, p = 0.94). Findings were similar when using the geometric mean IC50 value across the virus panel rather than breadth or potency scores (OR 1.00 [95% CI 0.99–1.01, p = 0.62] and VL-adjusted OR of 1.00 [95% CI 0.99–1.02, p = 0.92]), and when using alternative methods to score neutralization activity, such as that of Simek et al. (2009) (OR 1.20 [95% CI 0.04–3.67, p = 0.92] and VL-adjusted OR of 1.35 [95% CI 0.73–2.53, p = 0.33]) or factor analysis (OR 0.71 [95% CI 0.28–1.80, p = 0.47] and VL-adjusted OR of 0.99 [95% CI 0.74–1.32, p = 0.94]).

3.4. No Association Between SI Acquisition and Plasma and Cervical ADCC Activity

Fig. 2 and Table 2 summarize plasma and cervical ADCC activity in SI cases and controls, normalized to positive control (HIVIg) values. Seventy-six of 80 plasma samples (95%) and 70 of 83 cervical samples tested (84%) had detectable ADCC activity. In conditional logistic regression, no association was found between plasma ADCC activity and SI acquisition (OR 0.44, 95% CI 0.06–3.10, p = 0.41). Adjustment for contemporaneous viral load did not substantially alter the estimate (aOR 0.74, 95% CI 0.09–6.00, p = 0.78). Similarly, no association was found between cervical RFADCC activity and SI acquisition in either crude (OR 1.16, 95% CI 0.51–2.63, p = 0.73) or adjusted models (aOR 1.18, 95% CI 0.52–2.67, p = 0.25).

3.5. No Association Between SI Acquisition and Plasma IgG or IgA Binding to a Panel of Antigens

Fig. 2 and Table 2 summarize key pre-specified outcome measures in a screen of binding antibodies: IgG binding to each of three V1V2 scaffold antigens and IgA binding to gp140. Limited IgG binding to any of the V1V2 scaffold antigens was detected (Fig. 3a and Table 2). Of the 80 samples tested, detectable binding to 1FD6-Fc-ZM109_v1v2, 2J9C-ZM53_v1v2 and MuLVgp70-caseA2_v1v2 was observed in 24 (30%), 38 (48%) and 20 (25%) samples respectively (Fig. 3). When binding was averaged over the three variants, 42 (53%) of samples had detectable binding. In conditional logistic regression of dichotomized data (detectable vs. undetectable), no association was detected between SI acquisition and antibody binding to any single V1V2 variant or mean binding (Table 2): aOR for 1FD6-Fc-ZM109_v1v2 0.96, 95% CI 0.30–3.04, p = 0.94; aOR for 2J9C-ZM53_v1v2 0.97, 95% CI 0.26–3.63, p = 0.96; aOR for MuLVgp70-caseA2_v1v2 1.05, 95% CI 0.27–4.08, p = 0.94; aOR for average binding 0.64, 95% CI 0.20–2.06, p = 0.46. Crude and VL-adjusted estimates of the association were similar (Table 2).

In addition to IgG binding to V1V2 and IgA binding to gp140, IgG and IgA binding to a panel of additional exploratory antigens was evaluated (18 total antigens), including 5 gp120 variants, Env trimer, 4 V3 peptide variants (a consensus for each subtype), consensus A C1 peptide, 2 gp41 variants and the CD4 binding site core. No associations were found between any of the antigens examined and SI acquisition (Table 3).

3.6. No Interaction Between Humoral Responses

Interaction analysis evaluated whether the relationships between SI acquisition and either IgG-mediated ADCC activity or V1V2 IgG binding were modified by the level of gp140 IgG binding, as was observed in the RV144 analysis (Haynes et al., 2012). Conditional logistic regression did not demonstrate evidence of an interaction between either IgG-mediated immune function and gp140 IgA binding (Supplementary Table S2).

3.7. Sensitivity Analyses

We conducted two sensitivity analyses. The first included only samples ≥ 180 days after initial infection, at a time when immune responses would have had more time to mature, and at which greater evidence of protection from SI was observed in a prior study (Ronen et al., 2013). Twelve case-control sets were included this analysis. No associations were detected with any of the immune correlates assayed when restricting to samples ≥ 180 days after initial infection (Supplementary Table S3). The second sensitivity analysis included only intra-subtype SI cases, based on the env subtype, to enable comparison with studies of intra-subtype SI in other cohorts (Smith et al., 2006; Basu et al., 2012, 2014). Ten to twelve case-control sets were included in this analysis, based on sample availability of intra-subtype SI cases. No associations were detected with any of the immune correlates assayed (Supplementary Table S4).
4. Discussion

Elucidation of the immune responses that provide protection from HIV infection is an important step in designing protective vaccines. We have previously reported that the incidence of SI is two-fold lower than that of initial infection, after adjustment for differences in sexual risk behavior (Ronen et al., 2013), suggesting the immune response to initial infection may provide some protection against subsequent infection. In the present study, we employed a matched case-control study design to characterize differences between the immune response of individuals who acquired SI and that of individuals who remained singly infected despite ongoing HIV exposure. Association between SI status and a panel of measures of humoral responses against HIV was evaluated, including plasma IgG binding to V1V2, plasma IgA binding to gp140, plasma neutralizing antibody breadth and potency, and plasma and genital ADCC. We found no statistically significant association between SI acquisition and any of the antibody functions assayed. Interaction analysis did not find evidence of modification of the effect of IgG-mediated binding or killing by IgA binding. Sensitivity analyses restricted to individuals who had been HIV-infected for at least 6 months, and to intra-subtype SI cases, did not change these results. Exploratory analysis of 32 additional binding antibody activities did not suggest any additional associations with SI acquisition.

Our study hypotheses were motivated by the findings of several prior studies. Analysis of recipients of the RV144 vaccine pointed to IgA binding to gp140 and IgG binding to V1V2 as positive and negative correlates of HIV acquisition, respectively, with evidence of antagonism between the two functions (Haynes et al., 2012). Additionally, several antibody effector functions have been implicated as correlates of natural HIV infection. In the context of mother-to-child transmission, breast milk IgA binding to gp140 (Pollara et al., 2015) and ADCC activity (Mabuka et al., 2012) were associated with reduced breast milk trans..

**Table 2**

| Correlate                  | N (case, control) | OR   | 95% CI         | p-Value | aORa | 95% CI         | p-Value |
|---------------------------|-------------------|------|----------------|---------|------|----------------|---------|
| Plasma neutralization breadth | 13, 39            | 1.00 | 0.52–1.93      | 1.00    | 1.35 | 0.73–2.53       | 0.33    |
| Plasma neutralization potency | 13, 39            | 0.93 | 0.75–1.15      | 0.53    | 0.99 | 0.74–1.32       | 0.54    |
| Plasma ADCC activity      | 20, 60            | 0.44 | 0.06–3.10      | 0.41    | 0.74 | 0.09–6.00       | 0.78    |
| Cervical ADCC activity    | 17, 51            | 1.16 | 0.51–2.63      | 0.73    | 0.18 | 0.52–2.67       | 0.70    |
| Plasma V1V2 IgG binding   |                   |      |                |         |      |                |         |
| ZM109                     | 20, 60            | 1.00 | 0.31–3.18      | 1.00    | 0.96 | 0.30–3.04       | 0.94    |
| ZM53                      | 20, 60            | 0.89 | 0.24–3.33      | 0.80    | 0.97 | 0.26–3.63       | 0.96    |
| Case A2                   | 20, 60            | 1.00 | 0.27–3.69      | 1.00    | 1.05 | 0.27–4.08       | 0.94    |
| Average                   | 20, 60            | 0.79 | 0.48–1.29      | 0.34    | 0.64 | 0.20–2.06       | 0.46    |
| Plasma gp140 IgA binding  | 20, 60            | 0.93 | 0.58–1.49      | 0.78    | 0.85 | 0.51–1.41       | 0.52    |

ADCC = antibody-dependent cell-mediated cytotoxicity, aOR = adjusted odds ratio, CI = confidence interval, OR = odds ratio, SI = superinfection, VL = viral load.

a Dichotomized detectable vs. undetectable.

b Adjusted for VL.
we individually matched, potentially allowing for more complete adjustment for confounding by time since infection. This may be critical given that timing of sampling relative to initial infection and SI acquisition is an important determinant of the strength of the immune response. Indeed, we specifically excluded cases of SI that occurred in the first two months after initial infection because small differences between a case and its controls in the accuracy of infection timing could lead to spurious differences in neutralization activity when assaying early on in infection. Later in infection, neutralization activity would be expected to be less influenced by small differences in timing.

It is of interest to note that the Mombasa Cohort differs from the other SI cohorts studied in its inclusion of intra-subtype and inter-subtype SI cases. It is possible that certain immune correlates identified in previous studies are protective against antigenically similar viruses. Sensitivity analysis of our data restricted to 12 intra-subtype SI cases did not indicate associations with any of the immune functions assayed. Sample size limitations in this and previous studies make it impossible to reliably address this aspect; additional studies of comparable size to the current study in other cohorts or pooling of samples and data are needed to clarify these immune correlates in intra-subtype infections.

If there is some immune protection within clades but limited protection between clades, this would be highly relevant for vaccine design and implementation.

Our observation of no statistically significant associations between any of the immune responses assayed and acquisition of SI suggests that the protection from superinfection observed in our epidemiologic study of the Mombasa Cohort (Ronen et al., 2013) was not mediated by the humoral immune functions ascertained in this study. This finding has a number of explanations.

First, it is possible that the assays we performed did not adequately capture the immune deficit of individuals who acquire SI. Ours is the most comprehensive analysis of immune correlates of SI published to date, evaluating 5 primary humoral immune responses in plasma and genital samples, and over 30 additional exploratory binding antibody

![Fig. 3. Plasma binding antibody in superinfection cases and controls.](image)

### Table 3

| Binding correlate | OR   | 95% CI | p    |
|-------------------|------|--------|------|
| bg505 gp120 IgG   | 1.17 | 0.61–2.23 | 0.64 |
| c294 gp120 IgG   | 0.80 | 0.42–1.53 | 0.50 |
| b035 gp120 IgG   | 1.00 | 0.62–1.64 | 0.99 |
| zm109 gp120 IgG  | 0.98 | 0.56–1.72 | 0.94 |
| sf62 gp120 IgG   | 0.99 | 0.49–2.01 | 0.98 |
| conA gp140 IgG   | 2.74 | 0.72–10.43 | 0.14 |
| bg505 trimer IgG | 0.93 | 0.48–1.84 | 0.84 |
| conA1 v3 IgG     | 1.59 | 0.47–5.43 | 0.46 |
| conB v3 IgG      | 1.00 | 0.57–1.78 | 1.00 |
| conC v3 IgG      | 0.47 | 0.14–1.56 | 0.22 |
| conD v3 IgG      | 1.00 | 0.68–1.47 | 0.39 |
| conA C1 IgG      | 1.44 | 0.80–2.60 | 0.22 |
| mGp41 IgG        | 1.47 | 0.74–2.95 | 0.28 |
| za1197 gp41 IgG  | 1.51 | 0.65–3.61 | 0.34 |
| rsc3** IgG       | –    | –      | –    |
| zm109 v1v2 IgA   | 2.79 | 0.81–9.68 | 0.11 |
| caseA2 v1v2 IgA  | 1.69 | 0.47–6.13 | 0.42 |
| zm53 v1v2 IgA    | 1.24 | 0.86–1.79 | 0.26 |
| bg505 gp120 IgA  | 1.08 | 0.66–1.78 | 0.76 |
| c294 gp120 IgA   | 0.88 | 0.58–1.35 | 0.56 |
| b035 gp120 IgA   | 0.75 | 0.49–1.15 | 0.19 |
| zm109 gp120 IgA  | 1.00 | 0.69–1.44 | 0.99 |
| sf62 gp120 IgA   | 0.97 | 0.62–1.53 | 0.90 |
| bg505 trimer IgA | 0.72 | 0.41–1.26 | 0.24 |
| conA1 v3 IgA     | 1.59 | 0.47–5.43 | 0.46 |
| conB v3 IgA      | 2.31 | 0.57–9.46 | 0.24 |
| conC v3 IgA      | 1.45 | 0.42–4.99 | 0.56 |
| conD v3 IgA      | 2.06 | 0.89–4.80 | 0.09 |
| conA C1 IgA      | 1.47 | 0.60–3.58 | 0.40 |
| mgp41 IgA        | 1.24 | 0.50–3.06 | 0.64 |
| za1197 gp41 IgA  | 2.48 | 0.72–8.56 | 0.15 |
| rsc3 IgA         | 1.36 | 0.33–5.62 | 0.68 |

N = 20 cases, 60 controls for all analyses. CI = confidence interval, OR = odds ratio, SI = superinfection.

a Dichotomized detectable vs. undetectable.

b Model failed to converge due to too many undetectable observations (78 of 80).
activities using the same samples. Several of the activities assayed have not previously been characterized in studies of superinfection, including ADCC activity in genital samples or binding to gp140, V3, Env trimer, CD4 binding site or gp41. Nonetheless, our assays were limited. Use of a 4-virus panel for neutralization assays, though validated against a larger panel, may have reduced the assay's sensitivity. Moreover, non-humoral mechanisms of protection such as T-cell or NK-cell responses, or immune activation prior to SI, which can lead to increased target cells for HIV, were not assessed in this study and could contribute to SI acquisition risk, as suggested by a small prior study from the Mombasa Cohort (Blish et al., 2012). These results, coupled with the observed temporal association with SI acquisition we have previously reported (Ronen et al., 2013), prompt greater focus on these mechanisms. Second, unlike participants in the RV144 vaccine trial, in which all vaccinated were exposed to the same immune stimulus, each participant in the Mombasa Cohort was exposed to a different initial virus. These different antigens may have stimulated heterogeneous immune responses, which could make it more challenging to detect any single correlate. Third, ongoing HIV exposure could not be directly evaluated in singly infected controls. Unknown HIV exposure status in the singly infected controls, some of whom may not have been exposed, would be expected to yield an attenuated estimate of the true association between immune response and SI acquisition. Similarly, our study has no information on viral or host factors in the source partners that could contribute to infection outcome. While there is little biological support for immune responses in the source partner driving transmission outcomes in the setting of sexual HIV-1 transmission, this has been observed in the context of mother-to-child transmission, where ADCC activity in maternal breastmilk was associated with transmission to the infant (Mabuka et al., 2012). Fourth, while this is the largest set of SI cases published to date all available samples were included and individual 3:1 matching was employed to increase sensitivity, sample sizes were still relatively small (n = 13–20 SI cases per analysis). It remains possible that an association exists in our data that we were underpowered to detect. Pooled analysis of SI cases and controls from multiple cohorts could increase power to detect immune deficits. Finally, it is possible that the protection from SI acquisition previously observed in the cohort is not explained by immune protection, but by residual confounding by behavioral risk.

In conclusion, these findings do not provide evidence for a strong protective effect of humoral immune responses in the setting of natural exposure to diverse circulating HIV variants. This suggests that a protective HIV vaccine may need to elicit cellular immune mechanisms such as NK or T-cell responses, or a stronger humoral response than that seen in the naturally infected women included in our study.

Funding Sources
This work was supported by the National Institutes of Health, grant numbers R37 AI038518 to J.O. and T32 CA080416 to K.R. and A.S.D. The funders had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest
The authors have no conflicts of interest to declare.

Author Contributions
Designed the study: KR, ASD, SMG, RSM, JO. Performed the experiments: KR, ASD. Analyzed data: KR, ASD, SMG, RSM. Contributed reagents and materials: WJ, KM. Wrote the paper: KR, ASD, JO, with input from all authors.

Acknowledgements
We gratefully acknowledge to the participants of the Mombasa Cohort and staff at the study clinic. We thank Xiangpeng Kong and Xunjing Jiang for providing 2J9C and 1FD6-Fc V1V2 scaffold proteins.

Appendix A. Supplementary data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2017.04.005.

References
Altfeld, M., Allen, T.M., Yu, X.G., Johnston, M.N., Agrawal, D., Korber, B.T., Montefiori, D.C., O’Connor, D.H., Davis, B.T., Lee, P.K., Maier, E.L., Harlow, J.,oulder, P.J.R., Brander, C., Rosenberg, E.S., Walker, B.D., 2002. HIV-1 superinfection despite broad CSR + T-cell responses containing replication of the primary virus. Nature 420 (6914):434–439. http://dx.doi.org/10.1038/3501200.
Basu, D., Kraft, C.S., Murphy, M.K., Campbell, P.J., Yu, T., Hrabar, P.T., Irene, C., Pinter, A., Chomba, E., Mulenga, J., Klimente, W., Allen, S.A., Derdeyn, C.A., Hunter, E., 2012. HIV-1 subtype C superinfection in Zambia: evidence for low autologous neutralizing antibody responses prior to intrasubtype HIV-1 infection. Retrovirology 9:76. http://dx.doi.org/10.1186/1742-6469-9-76.
Basu, D., Xiao, P., Erde, Z., Berez, A., Britt, W.J., Mulenga, J., Klimente, W., Allen, S.A., Derdeyn, C.A., Hunter, E., 2014. HIV-1 subtype C superinfection in Zambians prior to HIV-1 subtype C superinfection. Virology 462-463:295–298. http://dx.doi.org/10.1016/j.virol.2014.06.016.
Blish, C.A., Dogan, O.C., Jaako, W., McClelland, R., Mandaliya, K., Odevem-Davis, K.S., Richardson, B.A., Overbaugh, J., 2008. Human immunodeficiency virus type 1 superinfection occurs despite relatively robust neutralizing antibody responses. J. Virol. 82 (4):12994–12103. http://dx.doi.org/10.1128/JVI.01730-08.
Blish, C.A., Dogan, O.C., Jaako, W., McClelland, R., Mandaliya, K., Odevem-Davis, K.S., Richardson, B.A., Overbaugh, J., 2012. Cellular immune responses and susceptibility to HIV-1 superinfection: a case-control study. AIDS 26 (5):643–646. http://dx.doi.org/10.1097/QAD.0b013e3285f0a9f6.
Blish, C.A., Jalalad-Lechak, Z., Rainwater, S., Nguyen, M.-A., Dogan, O.C., Overbaugh, J., 2009. Cross-subtype neutralization sensitivity despite monoclonal antibody resistance among early subtype A, C, and D envelope variants of human immunodeficiency virus type 1. J. Virol. 83 (15):7783–7788. http://dx.doi.org/10.1128/JVI.00673-09.
Blish, C.A., Nedellec, R., Mandaliya, K., Mosier, D.E., Overbaugh, J., 2007. HIV-1 subtype C envelope variants from early in infection have variable sensitivity to neutralization and to inhibitors of viral entry. AIDS 21 (6):893–897. http://dx.doi.org/10.1097/QAD.0b013e32805e8727.
Chohan, B., Lavreys, L., Rainwater, S.M.J., Overbaugh, J., 2005. Evidence for frequent re-infection with human immunodeficiency virus type 1 of a different subtype. J. Virol. 79 (16):10701–10708. http://dx.doi.org/10.1128/JVI.01078-05.
Cortez, V., Oden-Davis, K., McClelland, R.S., Jaako, W., Overbaugh, J., 2012. HIV-1 superinfection in women broadens and strengthens the neutralizing antibody response. PLOS Pathog. 8 (3):e1002611. http://dx.doi.org/10.1371/journal.ppat.1002611.
Cortez, V., Wang, B., Dingins, 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 (7):e1004973 Edited by R. C. Desrosiers. http://dx.doi.org/10.1371/journal.ppat.1004973.e008.
Forthal, D.N., Landucci, G., Chohan, B., Richardson, B.A., McClelland, R.S., Jaako, 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 (1):31–33. http://dx.doi.org/10.1097/QAI.0b013e3182784d41.
Fouda, G.G., Cunningham, C.K., McFarland, E.J., Borkowski, W., Muresan, P., Pollara, J., Soni, Y.V., Lieb, R.E., Whitaker, K., Shen, X., Vandergeift, N., Overman, K.G., Yates, N.L., Moody, M.A., Fry, C., Kim, J.H., Michael, N.R., Robb, M., Pitisuttithum, P., Kaewkungwal, J., Niyataphan, S., Kerks-Ngarm, S., Liao, H.-X., Haynes, B.F., Montefiori, D.C., Ferrari, G., Tomaras, G.D., Permar, S.R., 2015. Infant HIV type 1 gp120 vaccine elicits robust and durable anti-V1V2 immunoglobulin G responses and only rare envelope-specific immunoglobulin A responses. J. Infect. Dis. 211 (4):508–517. http://dx.doi.org/10.1093/infdis/jiu444.
Gómez-Román, V.R., Flores, R.H., Patterson, L.J., Peng, B., Venzon, D., Aldrich, K., Robert-Guroff, M., 2006. A simplified method for the rapid fluorometric assessment of antibody-dependent cell-mediated cytotoxicity. J. Immunol. Methods 308 (1):52–67. http://dx.doi.org/10.1016/j.jimmu.2005.09.018.
Gray, E.S., Madiga, M.C., Hermanus, T., Moeve, P.J., Whitter, C.K., Tumba, N.L., Werner, L., Misiana, K., Sibeko, S., Williamson, C., Abdool Karim, S.S., Morris, L., CAPRISA002 Study Team, 2011. 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. 85 (10):4826–4840. http://dx.doi.org/10.1128/JVI.00198-11.
Haynes, B.F., Gilbert, P.B., Mcclatchy, M.J., Zolla-Pazner, S., Deininger, S.A., Peterson, L.J., Patterson, L.J., Peng, B., Venzon, D., Aldrich, K., Robert-Guroff, M., 2006. A simplified method for the rapid fluorometric assessment of antibody-dependent cell-mediated cytotoxicity. J. Immunol. Methods 308 (1):52–67. http://dx.doi.org/10.1016/j.jimmu.2005.09.018.
HIV-1 vaccine efficacy trial. N Engl J Med. 366 (14):1275–1286. http://dx.doi.org/10.1056/NEJMoa1113425.

Jiang, X., Totrov, M., Li, W., Sampson, J.M., Williams, C., Lu, H., Wu, X., Lu, S., Wang, S., Zolla-Pazner, S., Kong, X.-P., 2016. Rationally designed immunogens targeting HIV-1 gp120 VIV2 induce distinct conformation-specific antibody responses in rabbits. J Virol. 90 (24):11007–11019. http://dx.doi.org/10.1128/JVI.01490-16.

Kraft, C.S., Hunter, E, Manigart, O., 2010. Identification of HIV superinfection requires longitudinal samples and sequence analysis. J. Infect. Dis. 1:32.

Li, M., Salazar-González, J.F., Derdeyn, C.A., Morris, L., Williamson, C., Robinson, J.E., Decker, J.M., Li, Y., Salazar, M.G., Polonis, V.R., Milana, K., Karim, S.A., Hong, K., Greene, K.M., Bliska, M., Zhou, J., Allen, S., Chomba, E., Mulenga, J., Wawula, C., Gao, F., Zhang, M., Korber, B.T.M., Hunter, E., Hahn, B.H., Montefiori, D.C., 2006. Genetic and neutralization properties of subtype C human immunodeficiency virus type 1 molecular env clones from acute and early heterosexually acquired infections in Southern Africa. J Virol. 80 (23):11775–11790. http://dx.doi.org/10.1128/JVI.01730-06.

Long, E.M., Rainwater, S.M.J., Lavreys, L., Mandaliya, K., Overbaugh, J., 2002. HIV type 1 variants transmitted to women in Kenya require the CCR5 coreceptor for entry, regardless of the genetic complexity of the infecting virus. AIDS Res. Hum. Retrovir. 18 (8):567–576. http://dx.doi.org/10.1089/08922207275374914.

Mabuka, J., Ndutu, O., Odeh-Davis, K., Peterson, D., Overbaugh, J., 2012. HIV-specific antibodies capable of AIDC are common in breastmilk and are associated with reduced risk of transmission in women with high viral loads. PLOS Pathog 8 (6):e1002739. http://dx.doi.org/10.1371/journal.ppat.1002739.

Martin, H.L., Nyange, P.M., Richardson, B.A., Lavreys, L., Mandaliya, K., Jackson, D.J., van der Horst, C., Kourtis, A.P., Tomaras, G.D., Ferrari, G., Permar, S.R., 2015. Association of HIV-1 Envelope-Specific Breast Milk IgA Responses with Reduced Risk of Perinatal HIV infection. J Infect. Dis. 178 (4):1053–1059. http://dx.doi.org/10.1093/infdis/jiu146.

Monte, H.-X., Brinkley, C., Denny, T.N., Ochsenbauer, C., Ellington, S., King, C.C., Jamieson, D.J., 2009. Breadth of neutralizing antibody response to human immunodeficiency virus type 1. J. Infect. Dis. 178 (4):1053–1059. http://dx.doi.org/10.1093/infdis/jiu146.

Redd, A.D., Mullis, C.E., Serwadda, D., Kong, X., Martens, C., Rickels, S.M., Tobian, A.A.R., Xiao, C., Grabowski, M.K., Nalugoda, F., Kigozi, G., Laeyendecker, O., Kagaayi, J., Sewankambo, N., Gray, R.H., Porcella, S.F., Weaver, M.J., Quinn, T.C., 2012. The rates of HIV superinfection and primary HIV incidence in a general population in Rakai, Uganda. J. Infect. Dis. 206 (2):267–274. http://dx.doi.org/10.1093/infdis/jis325.

Redd, A.D., Quinn, T.C., Tobian, A.A., 2013. Frequency and implications of HIV superinfection. Lancet Infect. Dis. 13 (7):622–628. http://dx.doi.org/10.1016/S1473-3099(13)70065-5.

Ronen, K., McCoy, C.O., Matsen, F.A., Boyd, D.F., Emery, S., Odeh-Davis, K., Jaako, W., Mandaliya, K., McClelland, R.S., Richardson, B.A., Overbaugh, J., 2013. HIV-1 superinfection occurs less frequently than initial infection in a cohort of high-risk Kenyan women. PLOS Pathog. 9 (8):e1003593. http://dx.doi.org/10.1371/journal.ppat.1003593.

Ronen, K., Richardson, B.A., Graham, S.M., Jaako, 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 (15):2281–2286. http://dx.doi.org/10.1097/QAD.0000000000000422.

Sanders, R.W., Derking, R., Cupo, A., Julien, J.-F., Yasmeen, A., de Val, N., Kim, J.H., Blattner, C., la Peña, de, A. T., Korzun, J., Golabek, M., de los Reyes, K., Ketas, T. J., van Gils, M. J., King, C. R., Wilson, I. A., Klasse, P. J. and Moore, J. P., 2013. A Next-Generation Cleaved, Soluble HIV-1 Env Trimer, BG505 SOSIP.664 gp140, Expresses Multiple Epitopes for Broadly Neutralizing but Not Non-Nutrelizing Antibodies. PLOS Pathog. 9 (8):e1003618 Edited by A. Trkola. http://dx.doi.org/10.1371/journal.ppat.1003618.s004.

Smith, D.M., Strain, M.C., Frost, S.D.W., Pillai, S.K., Wong, J.K., Wrin, T., Laufer, D.S., Lehrman, J.K., Boaz, M., Tarragona-Fiós, T., Miuro, G., Bruni, G., Pozniak, A., McPhee, D.A., Manigart, O., Karita, E., Inwoley, A., Jaako, W., Dehoitz, J., Bekker, L.-G., Pitisuttithum, P., Paris, R., Waller, L.M., Poignard, P., Wrin, T., Fast, P.E., Burton, D.R., Koff, W.C., 2009. Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. J. Virol. 83 (14):7337–7348. http://dx.doi.org/10.1128/JVI.00110-09.

Smith, D.M., Strain, M.C., Frost, S.D.W., Pillai, S.K., Wong, J.K., Wrin, T., Liu, Y., Petropoulos, C.J., Daar, E.S., Little, S.J., Richman, D.D., 2006. Lack of neutralizing antibody response to HIV-1 pre-diposes to superinfection. Virology 355 (1):1–5. http://dx.doi.org/10.1016/j.virol.2006.08.009.

Smith, D.M., Wong, J.K., Hightower, G.K., Ignoffo, C.C., Koelsch, K.K., Daar, E.S., Richman, D.D., Little, S.J., 2004. Incidence of HIV superinfection following primary infection. JAMA 292 (10):1177–1178. http://dx.doi.org/10.1001/jama.292.10.1177.

Tomaras, G.D., Ferrari, G., Shen, X., Alam, S.M., Lia, H.-X., Pollar, J., Bonsignori, M., Moody, M.A., Fong, Y., Chen, X., Poling, B., Nicholson, C.G., Zhang, R., Lu, X., Parks, R., Kaewkungwal, J., Niyapahan, S., Pitisuttithum, P., Reks-Ngarm, S., Gilbert, P.B., Kim, J.H., Michael, N.L., Montefiori, D.C., Hayes, B.F., 2013. Vaccine-induced plasma IgA specific for the C1 region of the HIV-1 envelope blocks binding and effector function of IgG. Proceedings of the National Academy of Sciences 110 (22):9019–9024. http://dx.doi.org/10.1073/pnas.1301456110.

Tomaras, G.D., Yates, N.L., Liu, P., Qin, L., Fouda, G.G., Chavez, L., Decamp, A.C., Parks, R.J., Ashley, V.C., Lucas, J.T., Cohen, M., Enon, J., Hicks, C.B., Liao, H.-X., Self, S.G., Landucci, G., Forthall, D.N., Weinhold, K.J., Keele, B.F., Hahn, B.H., Greenberg, M.L., Morris, L., Karim, S.S.A., Blattner, W.A., Montefiori, D.C., Shaw, C.M., Perelson, A.S., Hayes, B.F., 2008. Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia. J. Virol. 82 (24):12445–12463. http://dx.doi.org/10.1128/JVI.01708-08.

UNAIDS, 2016. Fact Sheet 2016. www.unaids.org 18 July. :pp. 1–8 Available at: http://www.unaids.org/sites/default/files/media_asset/UNAIDS_FinalFactSheet_en.pdf Accessed: 21 October 2016.

Williams, K.L., Cortez, V., Dingens, A.S., Gach, L.S., Rainwater, S., Weis, J.F., Chen, X., Spearman, P., Forthal, D.N., Overbaugh, J., 2015. HIV-specific CD4+induced antibodies mediate broad and potent antibody-dependent cellular cytotoxicity activity and are commonly detected in plasma from HIV-infected humans. ElBioMedicine 2 (10):1464–1477. http://dx.doi.org/10.1016/j.ebiom.2015.09.001.