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## Characterization of anti-HIV-1 neutralizing and binding antibodies in chronic HIV-1 subtype C infection

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### Abstract

Neutralizing (nAbs) and high affinity binding antibodies may be critical for an efficacious HIV-1 vaccine. We characterized virus-specific nAbs and binding antibody responses over 21 months in eight HIV-1 subtype C chronically infected individuals with heterogeneous rates of disease progression. Autologous nAb titers at study exit were significantly higher compared to contemporaneous responses at study entry ( $p=0.002$ ) and exit ( $p=0.01$ ). NAb IC<sub>50</sub> titers correlated inversely with V1-V2 length ( $p=0.04$ ). Significant differences in breadth and potencies were noted against subtype C compared to subtype A ( $p=0.03$  and  $p=0.01$ ) or subtype B ( $p=0.03$ ;  $p=0.05$ ) viruses respectively. IgG binding affinity for gp41 was higher than for gp120 ( $p=0.0002$ ). IgG-Fc $\gamma$ R1 affinity was significantly higher than Fc $\gamma$ RIIIa ( $p<0.005$ ) at study entry and Fc $\gamma$ RIIb ( $p<0.05$ ) or Fc $\gamma$ RIIIa ( $p<0.005$ ) at study exit. Evolving IgG binding suggests alteration of immune

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function mediated by binding antibodies. Evolution of nAbs was a potential marker of HIV-1 disease progression.

## Keywords

HIV-1 subtype C; neutralizing antibodies; binding antibodies; chronic infection

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## Introduction

In 2010 alone, there were an estimated 2.4 to 2.9 million new HIV-1 infections worldwide with 70% of these new infections occurring in sub-Saharan Africa (UNAIDS, 2011). This high HIV incidence makes the development of a protective vaccine a global public health priority. Such a vaccine will likely need to elicit antiviral antibodies, similar to the successful vaccines against other viral infections such as hepatitis B, measles, mumps and polio that are thought to mediate their effects primarily through antibody mechanisms (Plotkin, 2008). However, despite intense efforts in the study of HIV envelope structure and immunogen design, the development of an efficacious vaccine able to induce broadly effective antibody responses remains elusive.

During natural HIV infection, only a subset of individuals, less than 30%, develop broad, cross-neutralizing antibodies after many years (Gray et al., 2011; Li et al., 2009; Sather et al., 2009; Simek et al., 2009; Stamatatos et al., 2009). Such individuals have been an important source of new neutralizing monoclonal antibodies (nmAbs) against the HIV envelope that have enhanced our understanding of HIV pathogenesis, envelope structure and provided clues for rational immunogen design (Pejchal et al., 2011; Walker et al., 2010; Wu et al., 2010). However, clinical benefit of anti-HIV antibodies has not yet been definitively demonstrated. Given that HIV-1C is the predominant circulating and most rapidly spreading subtype worldwide (Esparza, 2005; Hemelaar et al., 2011), screening, characterizing and understanding the types of nAbs produced by HIV-1C-infected individuals; and defining the potencies and breadth of these nAbs may contribute to the design of the next generation of envelope immunogens.

While only some people develop cross-neutralizing antibodies, autologous nAbs (AnAbs) appear in almost all HIV-infected individuals usually within the first year. A number of studies have shown that contemporaneous viruses are less sensitive to AnAbs than earlier autologous viruses suggesting that viral evolution and escape occurs rapidly and this remains a significant obstacle to HIV vaccine development (Delwart et al., 1997; Moore et al., 2009; Richman et al., 2003; Rong et al., 2009; Wei et al., 2003). Several mechanisms of viral escape have been documented; these include insertions and deletions of amino acids, amino acid substitutions and shifting the position of N-linked glycans in Env (Frost et al., 2005; Lynch et al., 2011; Moore et al., 2009; Rong et al., 2009). Further understanding of HIV antibody escape patterns and mechanisms may help to inform better immunogen design to overcome Env diversity and immune escape.

Although the major focus of the HIV vaccine field is the development of immunogens able to induce broadly neutralizing antibodies, V1-V2 binding antibodies appear to have played some role against HIV-1 acquisition in the RV144 vaccine trial (Haynes et al., 2012). The role of binding or non-neutralizing antibodies in inhibiting virus replication through an Fc (fragment crystallizable) receptor (FcR)-dependent mechanism has been demonstrated (Peressin et al., 2011). FcRs are part of the immunoglobulin (Ig) superfamily and bind to the Fc portion of antibodies forming a bridge between the cell bearing the target antigens and the effector cell (Nimmerjahn and Ravetch, 2007). Fc $\gamma$ Rs have either an activating or

inhibitory function with relative IgG binding affinities. Fc $\gamma$ RI (high affinity), Fc $\gamma$ RIIa and Fc $\gamma$ RIIIa (medium-low affinity) - all have activating functions; and Fc $\gamma$ RIIb has an inhibitory function (medium-low affinity) (Forthal and Moog, 2009; Siberil et al., 2007). The effector cell subsequently mediates virus killing through antibody-dependent cell mediated cytotoxicity (ADCC) or through antibody-dependent cell-mediated viral inhibition (ADCVI). Some studies have shown that the strength of binding interaction between the Fc region of the antibody and the Fc $\gamma$ RIIIa and/or Fc $\gamma$ RIIa potently increases ADCC activity and Fc binding affinity could be altered through deglycosylation or site-specific mutagenesis, abrogating downstream ADCC (Jefferis, 2009; Lazar et al., 2006; Richards et al., 2008; Shields et al., 2001). Thus Fc-binding interaction may be crucial to improving the efficacy of antibodies (Lazar et al., 2006). These studies therefore provide a rationale for assessing the binding affinities of the IgG-Fc regions to their cognate Fc $\gamma$ Rs as an indication of putative antiviral activity of binding antibodies *in vivo*. Previously, ADCC activity was reported to be significantly higher in elite controllers compared to chronic progressors (Lambotte et al., 2009). These data on HIV are consistent with earlier studies suggesting a role for non-neutralizing antibodies in viral control and disease attenuation (Alsmadi et al., 1997; Baum et al., 1996). More recently, it was demonstrated that non-neutralizing antibodies conferred protection, albeit limited, to macaques against vaginal SHIV challenge (Burton et al., 2011). The binding affinities of IgGs to various HIV-1 antigens and to Fc $\gamma$ Rs have rarely been studied together and no studies have reported on the characterization of these antibodies in chronic HIV-1 subtype C infections.

In this study, we used standard high throughput neutralization assays to evaluate the potency and breadth of neutralizing activity in patient plasma and map potential Env targets associated with nAb breadth. We profiled autologous and cross-reactive (against a standard panel of 20 subtype A, B and C viruses) nAb titers in eight chronically HIV-1C infected individuals with heterogeneous rates of disease progression. Envelope genotypic characteristics (amino acid length and numbers of potential N-linked glycosylation sites (PNGs) associated with autologous nAb titers were investigated. Binding affinities of total immunoglobulins (IgGs) to HIV-1 antigens (gp41, gp120 and p24) and Fc $\gamma$ Rs (Fc $\gamma$ RI, Fc $\gamma$ RIIa, Fc $\gamma$ RIIb and Fc $\gamma$ RIIIa) were quantified. Associations between autologous nAb titers with markers of disease progression (namely CD4 T-cell counts and viral loads) were investigated.

## Materials and Methods

### Participants

Participant samples were retrospectively identified from the Sinikithemba cohort, which is a prospective natural history study of HIV-1 infected individuals based at McCord Hospital, Durban, South Africa as previously reported (Kiepiela et al., 2004). Human subjects for this study were eight chronically-infected individuals with heterogeneous rates of clinical disease progression as previously described (Archary et al, 2010). The median period of follow-up overall was 21 months. Ethical approval for this study was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee and all participants gave written informed consent to participate in the study.

### Sample Collection, CD4 T-cell counts and Plasma Viral Load

CD4 T-cell counts were performed at three-month intervals whereas viral loads were done at six-month intervals as previously described (Archary et al., 2010). Blood was drawn from each subject into EDTA tubes and plasma was separated by centrifugation and stored at  $-80^{\circ}\text{C}$  until use. Viral load was measured using the Amplicor Version 1.5 assay (Roche,

Alameda CA, USA). CD4 T-cell counts were enumerated by Trucount technology on a four colour FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA).

### Envelope clones

Briefly, single genome PCR amplification derived *env* amplicons were directionally T/A cloned into the CMV-driven expression plasmid pcDNA3.1-V5 HisTOPO-TA and screened for biological function as pseudoviruses following co-transfection with an *env*-deficient subtype B proviral plasmid (SG3ΔEnv) into 293T cells as described previously (Derdeyn et al., 2004; Haaland et al., 2009). A total of 20 standard reference envelope clones were obtained from the NIH AIDS Research and Reference Reagent Program and used in the heterologous neutralization assays. These included five subtype A, seven subtype B and eight subtype C envelope pseudoviruses as depicted in Table 2. Tier 1, 2 and 3 viruses were stratified previously based on a continuum of patterns of neutralization sensitivity- with tier 1A being the most sensitive, tier 1B above average, tier 2 displaying moderate to low sensitivity and tier 3 displaying the lowest sensitivity to neutralization (Seaman et al., 2010). The ConC plasmid based on the consensus of all the HIV-1 subtype C sequences from the Los Alamos database by 2001 was obtained from Dr Feng Gao (Kothe et al., 2006). The envelope plasmids containing single point mutations are described in Gray et al (2011).

### Neutralization assays

Patient plasma samples were evaluated for nAb activity against virions pseudotyped with autologous patient-derived viral Envs using a single reporter assay as described previously (Derdeyn et al., 2004; Gray et al., 2007; Li et al., 2005; Li et al., 2006; Wei et al., 2003). Figure 1A illustrates the schema used for autologous neutralizing antibody challenge assays where the study entry and exits Envs were tested for neutralization using the study entry and study exit plasma nAb samples for eight study participants. A total of 37 autologous Envs were tested against their study entry plasma and study exit plasma (range: 2-6 *env* clones per participant). Neutralization was measured as a reduction in luciferase gene expression after a single round of infection of TZM-bl cells (NIH AIDS Research and Reference Reagent Program). Two thousand infectious units of each pseudovirus was combined with five-fold dilutions of heat-inactivated participant plasma and incubated for 1 hour at 37°C as previously described (Rong et al., 2009). Subsequently, the virus and antibody reaction was added to the plated TZM-bl cells, and left to incubate at 37°C for 48 hours. The cells were then lysed and the luciferase activity was determined using a BioTek Synergy HT (BioTek, USA). The background luminescence of the uninfected wells was subtracted from the test wells. The percentage of infectivity was calculated by dividing the number of luciferase units at each plasma dilution by the value in the well containing no test plasma. The dilution that yielded 50% inhibitory activity against the virus, known as the nAb IC<sub>50</sub> titer was determined on Microsoft Excel. Each experiment was performed in duplicate and independently at least twice for replicability. The nAb IC<sub>50</sub> titer was calculated as the reciprocal plasma dilution causing a 50% reduction of relative light units (IC<sub>50</sub>). Heterologous assays were done as previously reported (Montefiori, 2004). Briefly, positive neutralization was scored as an IC<sub>50</sub> titer above 1:45. The nAb breadth and potency was determined as a score based on the median IC<sub>50</sub> titer for each virus as reported in Blish et al. (2008).

### Plasma IgG Isolation

Total IgGs were purified from the participant's plasma samples using the Melon Gel (Thermo Scientific, Surrey, United Kingdom) method according to the manufacturer's instructions. Briefly, 500 µl of a 1:10 dilution of plasma to buffer was added to the column. The tubes were rotated for 5 minutes in order to ensure maximum capture of the IgGs and were then spun down for 1 minute at 5,000 × g to elute the IgGs. IgG concentrations were

measured on the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Surrey, United Kingdom) and stored at 4°C for downstream ELISA assays.

### **FcGamma ( $\gamma$ ) Receptor Binding ELISAs**

Binding affinities of the bulk population of IgGs purified from plasma were measured for the various activating- Fc $\gamma$ RI, Fc $\gamma$ RIIa and Fc $\gamma$ RIIIa- (R&D Systems, Minneapolis, USA) and inhibitory receptors - Fc $\gamma$ RIIb.

Each ELISA plate (Ni-NTA HisSorb™ Plate Qiagen, Dusseldorf, Germany) was coated with 100  $\mu$ l/well of diluted Fc $\gamma$ R (Fc $\gamma$ RI, Fc $\gamma$ RIIa, Fc $\gamma$ RIIb, and Fc $\gamma$ RIIIa) at a concentration of 5  $\mu$ g/ml using PBS. One receptor type per plate was added and incubated overnight (O/N) at room temperature (RT). The plate was washed three times with PBS-Tween (0.05%) and blocked with 250  $\mu$ l/well 5% PBS-Bovine Serum Albumin (BSA) (Sigma-Aldrich, St Louis, USA) and then incubated for 1 hour at RT. The plate was washed three times with 0.05% PBS-Tween. 100  $\mu$ l/well of antibodies were added (the IgGs derived from the test plasma were diluted serially starting at 100  $\mu$ g/ $\mu$ l diluted down to 1.6  $\mu$ g/ $\mu$ l) to the respective wells and was left to incubate for 1 hour at RT. The plate was washed three times with 0.05% PBS-Tween. 100  $\mu$ l per well of anti-human IgG antibody, Fab fragment, peroxidase labeled was added (KPL Protein Research Products, Maryland, USA). 20  $\mu$ l of HRP Fab (1 mg/ml) was added to 10 mls PBS 1 $\times$  for one plate. The plate was incubated for 1 hour at RT and covered to protect it from light. The plate was washed three times with 0.05% PBS-Tween. The colour was resolved with 100  $\mu$ l/well of O-phenylenediamine (OPD) (Sigma-Aldrich, St Louis, USA) - one OPD tablet was added to 11 mls of StrepHRP substrate), the reactions were stopped by adding 100  $\mu$ l/well of 2.5N sulphuric acid (Sigma-Aldrich, St Louis, USA). The plate was read at 490 nm using the Tecan Sunrise 16039400 plate reader (Tecan Group, Switzerland). The optical density data was exported to Microsoft Excel worksheet to calculate the 50% effective concentration (EC<sub>50</sub>) of each batch of total IgGs needed to bind HIV-specific gp41, gp120 and p24 antibodies and various Fc $\gamma$ R using an ELISA-based assay. EC<sub>50</sub> ( $\mu$ g/ml) corresponds to the concentration of antibody needed to achieve half of the maximal binding to Fc $\gamma$ R. The EC<sub>50</sub> binding titer of the IgGs was used as a surrogate indicator of how strongly or poorly the IgGs' Fc portion may bind to the Fc $\gamma$ R to recruit effector cells like phagocytic (macrophages) or non-phagocytic (natural killer cells) cells to initiate either ADCVI or ADCC activity. Essentially, the higher the EC<sub>50</sub> the more antibody is needed for antiviral activity like ADCC or ADCVI to occur, the lower the affinity of antibody binding to the respective target cells bearing the antigens e.g. the Fc $\gamma$ Rs to antibodies to initiate effector functions.

### **Gp120, gp41, and p24 Binding ELISAs**

Binding affinities of the bulk population of IgGs purified from plasma were measured for the various HIV-1 specific antigens- gp120, gp41 and p24. ELISA plates were coated with 80  $\mu$ l/well of 250ng/ml gp120 (Immune Technology, New Jersey, USA) or gp41 (Immune Technology, New Jersey, USA) or p24 (Immune Technology, New Jersey, USA) and then incubated O/N at 4°C. The plates were washed three times with PBS Tween 0.05% and blocked with 5% PBS-BSA for 2 hours at RT (100  $\mu$ l/well). IgGs isolated previously were diluted in PBS and added to respective wells according to a ten times serial dilution to respective wells and incubated for 2 hours at RT. The plates were washed three times with 0.05% PBS Tween. 100  $\mu$ l anti-human IgG antibody, Fab fragment, peroxidase labeled was added to each well (KPL Protein Research Products, Maryland, USA) and left for 1 hour at RT (1:500 diluted-per plate: 20  $\mu$ l HRP in 10 ml PBS) and covered in tinfoil. The plate was washed 3 times with 0.05% PBS-Tween. One OPD tablet was added to 11 mls of phosphate citrate buffer + 4.4  $\mu$ l Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>); and 50  $\mu$ l/well were added. The reaction

was stopped by adding 50  $\mu$ l 2.5 N H<sub>2</sub>SO<sub>4</sub> per well. The plate was read at 490 nm on a microplate reader and the data analyzed using GraphPad Prism 5 software.

### Statistical analyses

Comparisons of different parameters including nAb IC<sub>50</sub> titers for both the autologous and heterologous responses, and binding affinities of the various HIV-specific antigens and Fc $\gamma$ Rs to IgGs was performed. Intra-group comparisons between study entry and study exit time-points were done using the Mann-Whitney U test and the Spearman rank correlation test. ANOVA was used to compare three or more groups and the Dunn's post-test was used to perform pairwise comparisons. A general linear model was used to estimate the rate of change in viral load and CD4 T-cell count over time. Viral load was log-transformed to ensure normality. All reported *p* values are two-sided and are considered significant if less than 0.05. The statistical analysis was performed using GraphPad Prism 5 and SAS version 9.3 (SAS Institute Inc., Cary).

## Results

### Study Participants

There were eight participants in this study, seven female and one male. Table 1 depicts the clinical profiles of the participants for this retrospective study. All participants were of African descent. All participants were antiretroviral treatment naïve for a median of 21 months. The median age was 31 years (range: 22-59 years). The median CD4 T-cell count at study entry was 578 cells/ $\mu$ l (range: 503-936 cells/ $\mu$ l) and at study exit was 369 cells/ $\mu$ l (range: 218-881 cells/ $\mu$ l). The median viral load at study entry was 6,140 copies/ml (range: 2,210-14,360 copies/ml) and at study exit was 14,750 (range: 2,440-345,000 copies/ml). The mean CD4 T-cell decline rate was  $-7$  cells/ $\mu$ l per month (standard error 1.50 cells/ $\mu$ l). Since data on the length of time that participants were infected was unavailable, sequence analysis was performed by using the Bayesian evolutionary analysis by sampling trees (BEAST) to estimate the approximate time of infection in both groups of participants. Participants were estimated to be infected for a mean period of 5.1 years (range: 9 months-15 years).

### Autologous Neutralizing Antibody Responses become potent over time in chronic infection

To better understand autologous neutralization during chronic infection we tested the participants' plasma against their plasma-derived Envs using a pseudovirus-based assay (see Figure 1A). The titers represented on Figure 1B were the average nAbIC<sub>50</sub> titers of two or three Envs per participant.

nAb IC<sub>50</sub> titers for the study entry and study exit Envs differed when the study entry and study exit plasma antibody responses were compared. As shown in Figure 1B, there were significant differences when the study entry Envs were tested against the study exit plasma (median nAb IC<sub>50</sub> of 1,178; range: 610-2,286) compared to the contemporaneous responses at study entry (median nAb IC<sub>50</sub> of 429; range: 119-628; *p*=0.002) and study exit (median nAb IC<sub>50</sub> of 305; range: 210-1,076; *p*=0.01). There were no correlations between the rates of CD4 T-cell decline or viral loads with for the nAb IC<sub>50</sub> titers of study entry Envs against the study exit plasma or between contemporaneous nAb IC<sub>50</sub> titers at study entry or study exit- Figure 1C and 1D.

### Autologous neutralization titers correlated with the length of the hypervariable regions V1-V2

Previous studies have shown an inverse association between neutralizing antibody titers and length of variable loops (V1-V2) and numbers of potential N-linked glycosylation sites

(PNGs) (Chackerian et al., 1997; Gray et al., 2007; Pinter et al., 2004; Rong et al., 2007; Sagar et al., 2006). To better understand the relationship between genotypic characteristics and neutralization sensitivity, the average amino acid length of the hypervariable loops of Env and PNGs were correlated to average autologous nAb IC<sub>50</sub> titers (Figure 2). Significant inverse correlation was found between the lengths of V1-V2 and nAb IC<sub>50</sub> titers ( $r=-0.80$ ;  $p=0.02$ ) for the entry viruses tested against the study exit plasma. There was a trend towards C2-V5 length correlation with nAb IC<sub>50</sub> titers ( $r=-0.67$ ;  $p=0.08$ ) and no significant correlations for V1-V5 ( $r=0.52$ ;  $p=0.2$ ). The same analysis was extended for nAb IC<sub>50</sub> titers and the numbers of potential N-linked glycosylation sites (PNGs) in the various hypervariable loops and there were no significant correlations observed.

### Neutralization breadth scores and potency scores indicate subtype-specific responses in chronic infection

To assess neutralization breadth in this chronic infection cohort, we investigated changes in plasma neutralization activity over a median of 21 months. Figure 3 depicts the profile of plasma neutralizing activity against a panel of heterologous Envs for the eight study participants at study entry and study exit. A total of 20 reference pseudoviruses from subtype A, B and C (as depicted in Table 2) were used to determine breadth and potency scores of neutralization according to the criteria previously described (Blish et al., 2008). Results are presented as a breadth score per subtype virus panel, and a total breadth score including all the subtype A, B and C viruses. Results are presented as potency scores per subtype, and total potency scores including all the subtype A, B and C viruses.

There was a wide range of variation in neutralization titers with most of the sera from the participants displaying preferential heterologous activity against the subtype C panel (Figure 3). Significantly higher breadth scores were observed against subtype C viruses (median: 3; range: 1-7) compared to subtypes A (median: 2; range: 0-4;  $p=0.03$ ) or B (median: 2; range: 0-6;  $p=0.01$ ) - Figure 3. Likewise, significantly higher potency scores were observed against subtype C viruses (median: 7.6; range: 0-152.1) compared to subtypes A (median: 4; range: 0-13.8;  $p=0.03$ ) or B (median: 3.9; range: 0-26.9;  $p=0.05$ ). Three of the participants (SK200, SK221 and SK233) showed a consistent increase of neutralization breadth scores and potency scores from study entry to study exit for subtypes C, B and A respectively translating into overall increase in total breadth and potency scores as well. Analysis of these participants indicated significantly higher viral load at study entry compared to study exit ( $p=0.04$ ); suggesting that increasing breadth may be a result of higher antigenic stimulation.

SK200 displayed the highest breadth scores and also had potent nAb IC<sub>50</sub> scores (Figure 3) to six of the eight subtype C viruses tested including five of six tier 2 viruses. SK200 had a total neutralization breadth score of 16 (range: 2-16) and the overall potency score was 177.1 (range: 3-177.1) at study exit. SK200 neutralized Subtype C- tier 2 viruses - CAP45.G3 and CAP239.G3 at study entry and exit plasmas, and CAP45.G3 in particular, was neutralized at nAb IC<sub>50</sub> titers of  $>2,000$  (range: 45-5,326). SK200 also displayed the highest titers  $>1,000$  at study entry (range: 45-1,316) and study exit (range: 45-1,829) to a subtype A tier 1 virus- Q23ENV17.

### CD4 T-cell count and viral load are not correlated with neutralization breadth

A number of studies have shown an inverse relationship between neutralization breadth and CD4 T-cell counts and a positive correlation with viral loads (Euler et al., 2010; Gray et al., 2011; Piantadosi et al., 2009; Sather et al., 2009). To better understand the relationship between neutralization and clinical disease markers in this subtype C chronic cohort, we investigated the association of CD4 T-cell counts and viral loads with neutralization breadth or potency over a median of 21 months. However, there were no significant correlations

between CD4 T-cell counts or viral loads and total breadth and potency scores (data not shown). A larger sample size may have been able to detect significant correlations between breadth or potency and markers of disease progression.

### Mapping of potential epitopes targeted by cross-neutralizing antibodies

The putative targets of neutralization in participants displaying the highest breadth scores for neutralization at study exit (SK200, SK221 and SK233) against the reference panel viruses were further investigated (using study exit plasma samples). Single point mutations in V2 (N160A, K165E or L165A) and C3 (N332A) were introduced into CAP45.G3, ConC, Du156.12, TRO.11 and Q23.17 and tested for loss of sensitivity against the three plasmas.

As depicted in Table 3, 5.6-fold and 4-fold drops in neutralization titers (relative to the wild type neutralization titers) were seen when plasma samples from SK221 and SK233, respectively, were tested against the K169E mutation in the CAP45 backbone. This suggested that the broadly neutralizing antibodies in these two participants targeted the V2 region and that a charge change from a lysine (K) to glutamic acid (E) at position 169 resulted in a disruption to the nAb epitope. In addition SK221 plasma also showed a 4.2 fold drop in neutralization titers to N332A made in the TRO.11 envelope indicating that the asparagine (N), or perhaps a potential N-linked glycosylation at this position, was essential for the antibody activity. Together, these results suggested that SK221 most likely had cross-neutralizing antibodies that target the V2 and C3 regions on Env. SK200, who displayed the highest neutralization breadth score, neutralized the wild-type and mutant viruses equivalently suggesting that this particular participant may have antibodies directed to other regions of Env, a profile that warrants further investigation.

### HIV-specific IgG binding titers for gp41, gp120 or p24 in chronic infection over 21 months

We isolated total immunoglobulins (IgGs) from study participants at study entry and study exit time points and measured the specificity and the binding affinity of IgGs to HIV-1 specific antigens. There were no significant differences in the median EC<sub>50</sub> binding affinities between study entry and study exit for gp41, gp120 or p24. Interestingly, at study entry, the median IgG binding affinities for gp120 (median: 137; range: 11.3-153.0) was significantly lower than for gp41 (median: 96.8; range: 71.9-112.1;  $p=0.0002$ ). Binding affinity for gp120 compared to p24 trended to be higher (median: 154.3; range: 57.9-293.9;  $p=0.09$ ) - Figure 4. These findings may suggest that binding IgGs were selectively binding gp41 compared to gp120 during the chronic infection stage. In addition, there were no significant correlations between the CD4 T-cell counts or viral loads at study entry or exit with the EC<sub>50</sub> of p24, gp120 or gp41 over the study period.

### IgG binding affinities differ for the various FcγR I, IIa, IIb and IIIa

To determine if the binding affinities of the IgGs with various activating and inhibitory FcγRs were different, we measured and compared the EC<sub>50</sub> binding titers over time between study entry and study exit time-points, and overall (Figure 5A). IgG binding for FcγRI at study entry was significantly higher than for FcγRIIIa ( $p<0.0005$ ). Likewise at study exit, the binding affinity of IgG for the FcγRI were consistently higher compared with FcγRIIb ( $p<0.05$ ) and FcγRIIIa ( $p<0.0005$ ). Significantly higher binding affinities for FcγRIIa at study entry were noted when compared to FcγRIIIa at study entry ( $p<0.05$ ). Likewise at study exit, the binding affinity of IgG for the FcγRIIa was higher compared to FcγRIIIa ( $p<0.05$ ) in chronic HIV-1 C disease. Collectively, these results indicated that there was differential kinetics of binding affinities of the IgG during chronic infection, which may then impact downstream non-neutralizing activity through ADCC. Sustained high affinity IgG binding to FcγRI may be preserved during chronic infection. There were

no significant correlations between viral loads or CD4 T-cell counts with the EC<sub>50</sub> IgG binding titers to the various receptors (FcγR I, IIa, IIb and IIIa)- Figures 5B and 5C.

## Discussion

Neutralizing and binding antibodies may play a role in HIV disease progression but these parameters have rarely been investigated concurrently. We therefore undertook this study in individuals chronically infected with HIV-1 subtype C, the most abundant subtype worldwide, to characterize neutralizing and binding antibody patterns in chronic disease, and to determine whether certain genotypic *env* characteristics are associated with autologous or heterologous nAb responses. Our data indicated that nAbs did not appear to protect against disease progression, rather, greater type-specific neutralization breadth and potency against subtype C Envs and increasing autologous nAb titers were associated with chronic disease progression. NAb IC<sub>50</sub> titers were correlated with *env* genotypic characteristics, including increased amino acid length in hypervariable regions V1-V2 of gp120.

In this study, nAb potency or breadth did not predict disease progression rate in individuals with chronic HIV-1 subtype C infection. However, a number of interesting findings were apparent. Firstly, high-level neutralizing titers to contemporaneous autologous virus were not observed in most of the participants. Instead, we observed significantly higher autologous responses over time (when the study entry viruses were tested against the study exit plasma) compared to contemporaneous responses, which suggests that nAb are continuously evolving during chronic infection. These findings also argue that an increased nAb titer *per se* is not effective at attenuating disease progression but rather is a marker of disease progression. Secondly, autologous nAb IC<sub>50</sub> titers correlated inversely with longer amino acid length for V1-V2 length indicating that increased length in this domain may be masking key neutralization epitopes. Indeed, evidence for V2 dependent epitopes was observed in SK221 and SK233. This data suggests that V1-V2 of Env may be persistently targeted by nAb in the chronic infection stage. Alternatively, there may be other intrinsic genetic differences of *env* that dictate neutralization potency and breadth that need to be further defined. Lastly, the dynamic nature of IgG binding affinity to various FcγRs and the selective high affinity binding to HIV-1 gp41 may be indicative of chronic disease progression.

The selective increase in neutralization breadth over time suggests that this parameter could be a marker of disease progression in chronic subtype C infection (Euler et al., 2010). Neutralization breadth did not significantly correlate with markers of disease progression (viral loads and CD4 T-cell counts in this chronic HIV-1C infection cohort, although the small sample size and short time-frame may have severely limited our statistical power to address this issue. Gray and colleagues (2011) found significant correlations between CD4 T-cell count and viral load with neutralization breadth at six months post-infection only and not at later time points (Gray et al., 2011). These observations suggest that higher antigenic stimulation may dictate the breadth of heterologous antibody responses (Doria-Rose et al., 2010; Fraser et al., 2007; Goujard et al., 2006; Mellors et al., 1997; Piantadosi et al., 2009; Sather et al., 2009). Although increased neutralization antibody breadth may not be protective against disease progression, they may be effective against super-infection as has been suggested by some studies (Smith et al, 2006 and Deeks et al, 2006), although conflicting data exists (Blish et al., 2008). Together, these studies have implications for HIV vaccine design, as vaccine immunogens may need to be given over long periods of time to stimulate the B cell response, and to facilitate affinity maturation which appears to be necessary for antibodies to acquire cross-neutralizing activity (Pancera et al., 2010; Scheid et al., 2009). For the participants who displayed potent cross-neutralizing antibody responses, their nAbs likely targeted quaternary V2 epitopes similar to PG9/PG16-like

antibodies (Moore et al., 2011). In addition, nAbs that targeted the N332 glycan in C3 suggest that the asparagine in that position is essential for neutralization activity. In subtype C infection, the V1-V2 and C3 regions are the immunodominant regions commonly targeted by AnAbs particularly during the early stage of infection (Lynch et al., 2011; Moore et al., 2008; Moore et al., 2009; Rong et al., 2009). Our results indicate the V1-V2 and C3 regions remain immunodominant and the focus of the nAb response resulting in broadly cross-neutralizing antibodies even during chronic progressive HIV-1 disease.

A decline of p24 antibody titers as disease progresses has previously been reported (Allain et al., 1987; Binley et al., 1997; Forster et al., 1987; Lange et al., 1986), and it is therefore not surprising that the p24-specific IgGs may lose their affinity over time. Chargelegue and colleagues found that low p24 IgG affinity correlated with HIV-1 disease progression (Chargelegue et al., 1995). Low affinity IgG HIV-1 specific binding antibodies may be indicative of chronic disease progression. It is plausible that a relative decrease in the binding affinity for activating Fc $\gamma$ RIIa and Fc $\gamma$ RIIIa may be consequent to changes in the Fc portion of the IgGs which do not affect Fc $\gamma$ RI binding. Polymorphisms in the Fc $\gamma$ RIIa (Forthal and Moog, 2009), and the extent of glycosylation of the antibodies (Forthal et al., 2010) affect binding affinity. Additionally, a decrease in binding affinity of Fc $\gamma$ RIIa was associated with dysfunction in the expression of these receptors on the cells of the innate immune system (Dugast et al., 2011). Dugast and colleagues purport that HIV infection is associated with a number of changes in FcR expression on phagocytic cells that are associated with changes in their ability to respond to antibody-opsonized targets, leading to a failure in viral clearance in chronic infection (Dugast et al., 2011). We found a consistently high affinity binding of IgG to Fc $\gamma$ RI over time which may suggest that the non-neutralizing pathway augmenting effector cell activity may be maintained during the chronic infection stage. However, in the absence of a functional assay, our data do not provide definitive answers on whether high affinity binding IgG to Fc $\gamma$ RI is biologically significant in chronic infection and what the profiles of the various Fc $\gamma$ R-IgG binding were during the initial HIV-1 infection stages.

Some limitations of our study are worth noting. Firstly, we only tested a limited number of viral clones from each patient, and therefore may have biased the results of the study to the cloned variants only. Secondly, we do not know the exact time of infection for the study participants and relied on short-term median (21 months) follow-up immunological data, which may be an unrepresentative snap-shot of the entire natural history of disease progression for these participants. Thirdly, the small sample size meant that our study was underpowered to reach definitive conclusions regarding the significance of breadth of neutralization and autologous responses in attenuating disease. Fourthly, we used binding affinity assays as an indication of the strength of affinity of the Fc portion of IgG to the Fc $\gamma$ Rs which may affect downstream *in vivo* effector cell activity. Lastly, the dynamic nature of IgG binding affinity to various Fc $\gamma$ Rs and the selective high affinity binding to HIV-1 gp41 may be indicative of differences and evolution of the antibody mediated innate antiviral immune response in progressive HIV-1 subtype C infection, although this will require additional functional assays to prove.

Despite these caveats, the findings suggest that an ongoing *de novo* nAb response does not directly protect against disease progression during chronic HIV-1 subtype C infection, corroborating data from previous studies. Our results are also consistent with data from non-human primate studies showing that high titer neutralizing antibody titers are not found in sooty mangabeys (Li et al., 2010) suggesting that autologous nAb are not part of the protection against disease progression in HIV and SIV infections. Furthermore, overall nAb breadth increased over time in most subjects, regardless of their disease status. The mechanism by which nAb breadth increased in these subjects is of interest. Thus, it will be

important to determine which Env epitopes in chronically infected individuals elicit broadly neutralizing antibodies and whether these provide any clinical benefit to the patient. In addition, animal studies suggest that anti-HIV antibodies can protect against HIV-1 infection (Burton et al., 2011; Hessel et al., 2009; Hessel et al., 2010) and vaccine-induced V1-V2 binding antibodies may be able to prevent infection (Haynes et al., 2012). Future studies will need to address whether neutralizing and binding antibodies from HIV infected individuals may be used to halt or reduce HIV acquisition.

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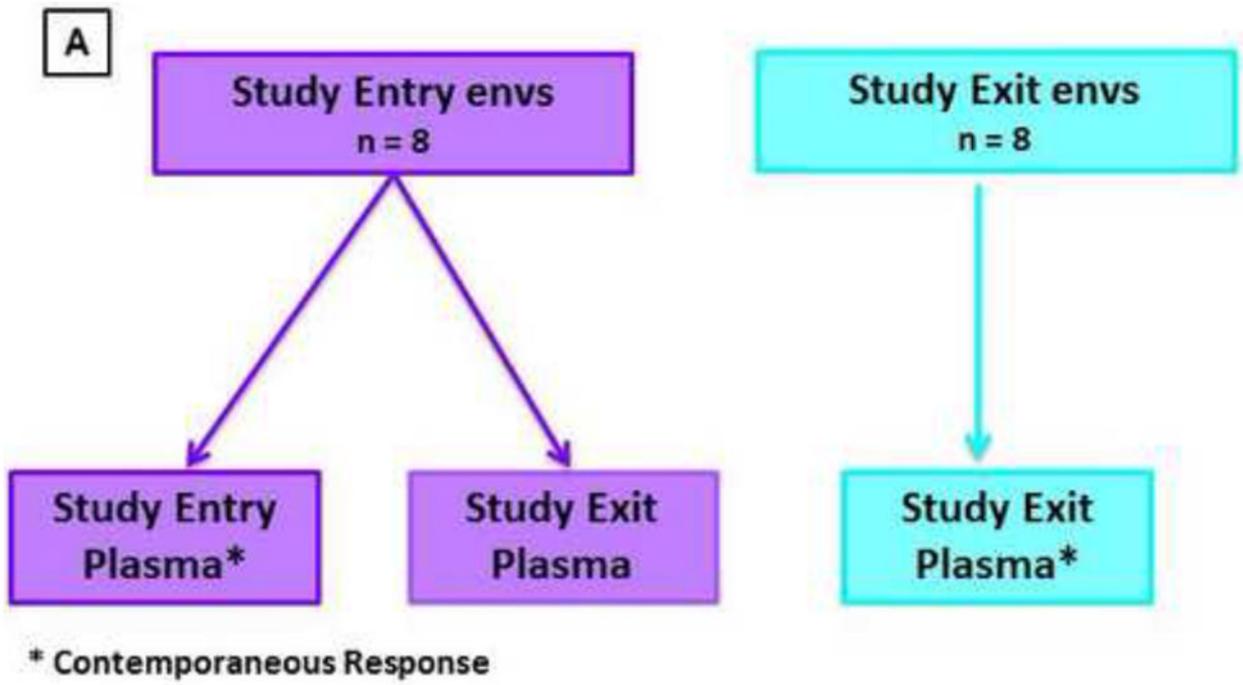
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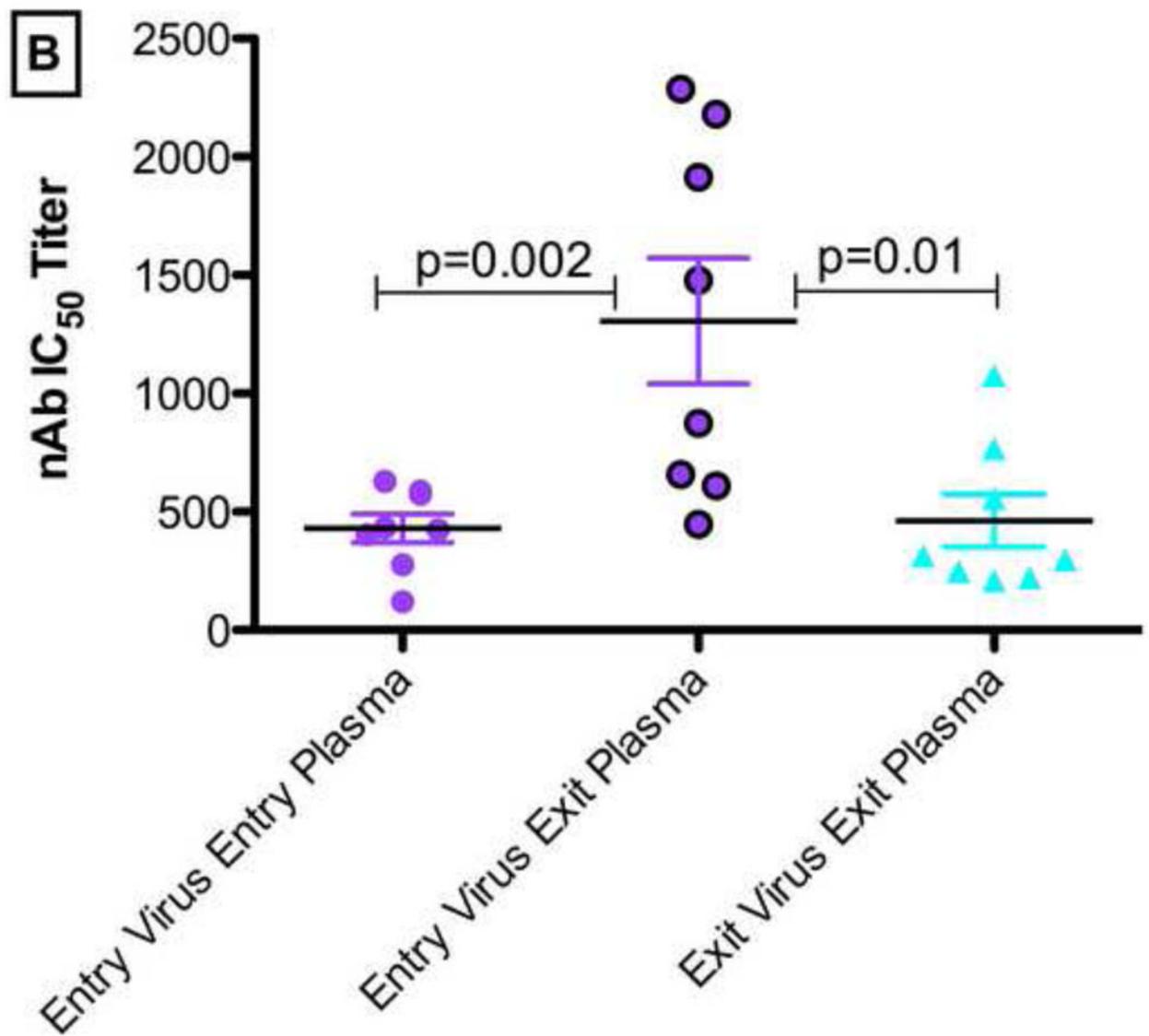
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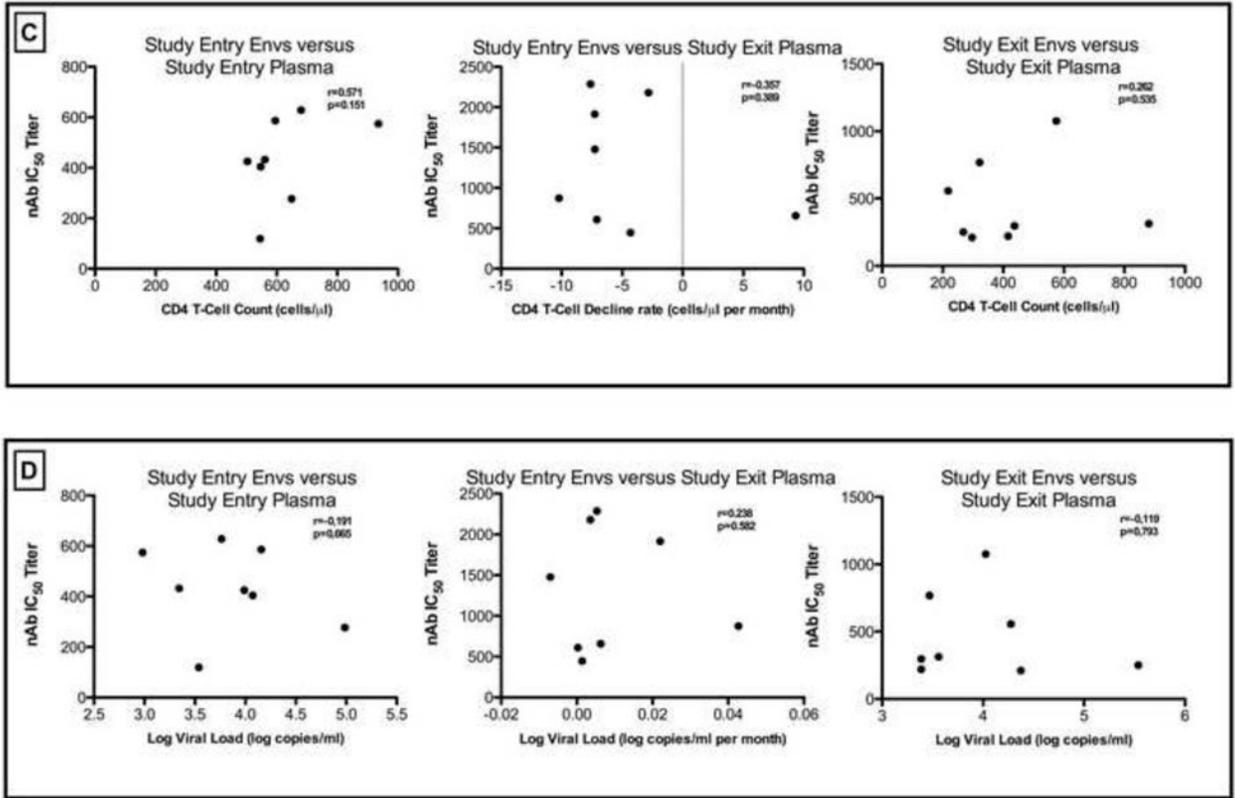
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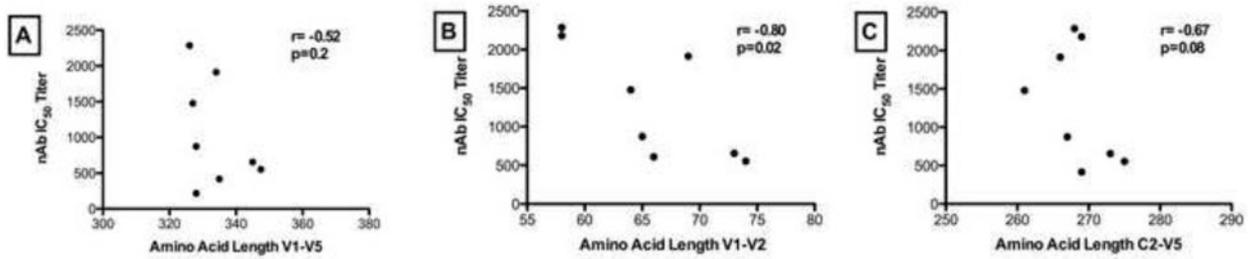






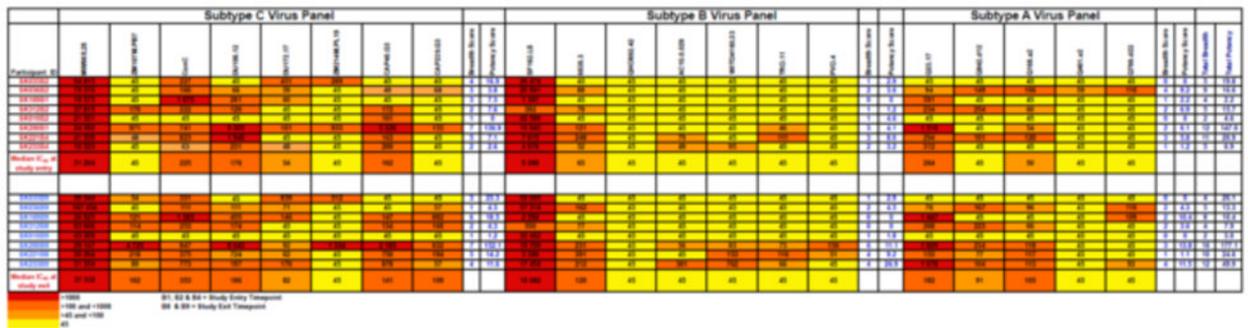
**Figure 1.**

(A). Schematic illustrating the autologous neutralizing antibody challenge assays in all participants over a median of 21 months from study entry to study exit. The average nAb IC<sub>50</sub> titers for study entry and exit Envs used in the assays are shown. The study entry Envs were tested against nAbs from the study entry (contemporaneous) and study exit plasma samples. Likewise the study exit Envs were tested against nAbs from the study entry and study exit (contemporaneous) plasma samples. Figure 1(B) depicts the autologous nAb IC<sub>50</sub> titers in study participant entry and exit plasma samples for all participants. Figure 1(C) and 1(D) shows the CD4 T-cell decline rate and the log viral load changes over time respectively. P-values <0.05 were considered significant. P value was calculated using the two-tailed Mann-Whitney non-parametric test overall. All the p values ( $p<0.0125$ ) remained statistically significant after Bonferroni adjustment for multiple comparisons.

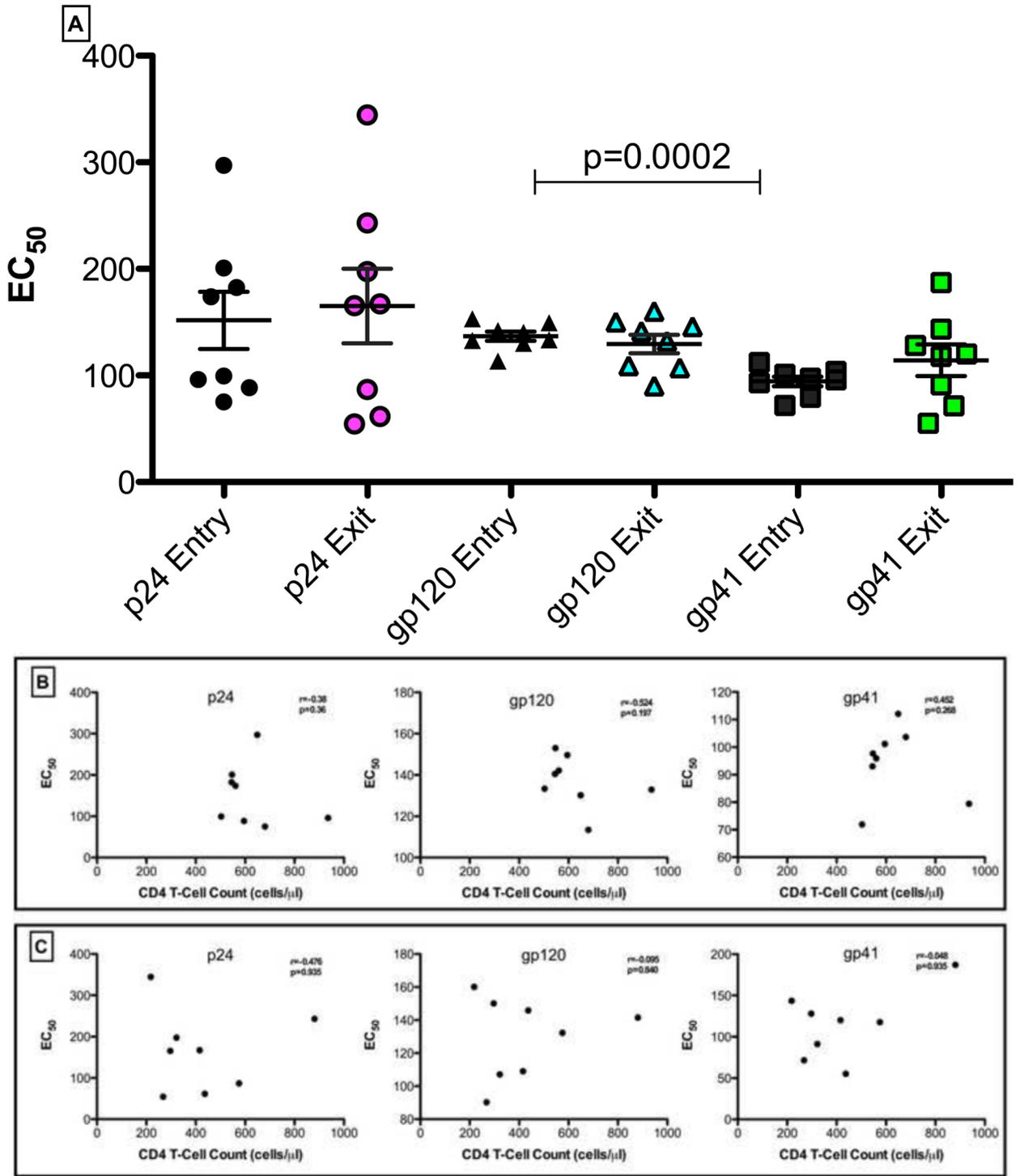


**Figure 2.**

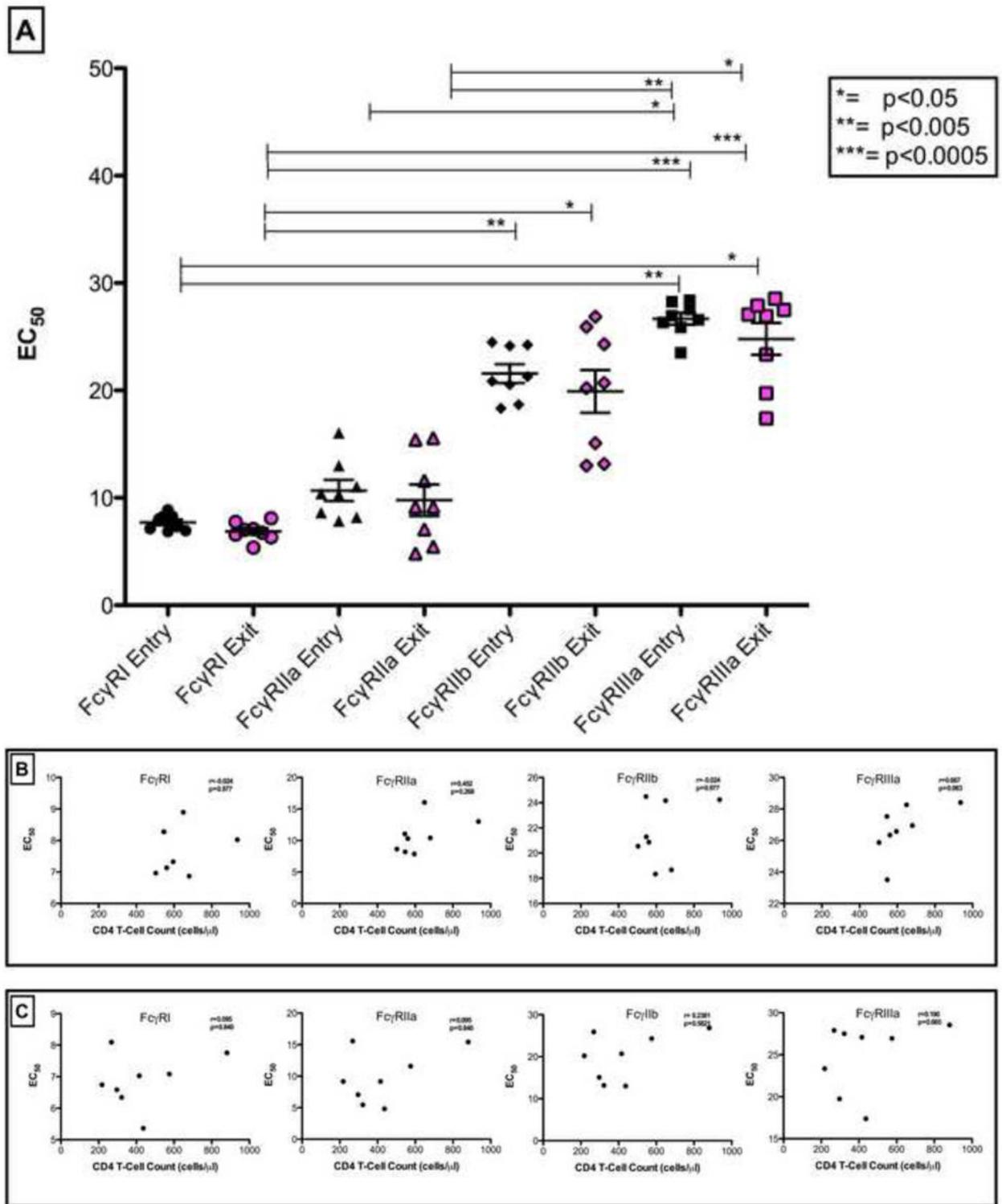
Correlation between the amino acid length of (A) V1-V5, (B) V1-V2 and (C) C2-V5 of Env and autologous neutralization titers (nAb IC<sub>50</sub> titer) when study entry viruses were tested against study exit plasma samples. The Spearman r-coefficient and p-values are shown and a correlation was considered significant when  $p < 0.05$ .



**Figure 3.** Heterologous responses of participant plasma tested at study entry and study exit against 20 Env pseudoviruses from a standard reference panel including subtypes C, B and A of tier 1A, 1B, tier 2 and 3 categories over a median of 21 months. The neutralization titer is shown as reciprocal plasma dilution required to inhibit 50% of virus infectivity when the virus is challenged with the participant's plasma. The highest titer (>1,000) is shown in red, and the lowest in light orange, yellow depicts a titer of <1:45 that is below detection as shown above.



**Figure 4.** (A). IgG EC<sub>50</sub> binding titers for gp41, gp120 and p24-specific antibodies at study entry and exit time points. Panels for Figure 4(B) and (C) show the correlation of EC<sub>50</sub> binding titers for gp41, gp120 and p24-specific antibodies with CD4-T cell counts at study entry and study exit respectively. None of the correlations were statistically significant ( $p < 0.05$ ).

**Figure 5.**

(A). EC<sub>50</sub> binding titers of IgGs for FcγRI, FcγRIIa, FcγRIIb and FcγRIIIa at study entry and exit time points. Intra- or inter-group comparisons were statistically different i.e.  $p < 0.05$ . Figure 5(B) and (C). Correlation of CD4 T-cell counts at study entry and study exit

with Fc $\gamma$ RI, Fc $\gamma$ RIIa, Fc $\gamma$ RIIb and Fc $\gamma$ RIIIa (EC<sub>50</sub>) are shown. Only p-values <0.05 are shown.

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**Table 1**

Clinical description of study participants.

Patient Identification	Sex	Age at Baseline	Viral Load study entry (copies/ml)	Viral Load study exit (copies/ml)	CD4 T-cell count study entry (cells/ $\mu$ l)	CD4 T-cell count study exit (cells/ $\mu$ l)	CD4 T-cell decline rate (cells/ $\mu$ l per month)
SK010	F	31	6,480	345,000	649	268	-10
SK035	F	31	5,800	2,950	680	322	-7
SK036	M	32	5,100	10,600	936	575	-7
SK169	F	30	2,210	2,440	561	437	-4
SK200	F	40	14,360	24,900	595	416	-7
SK221	F	30	9,740	23,700	503	297	-3
SK233	F	22	11,800	18,900	547	218	-8
SK312	F	59	3,460	3,630	545	881	-9

**Table 2**  
**HIV-1 Env pseudovirus panel of subtype C, B and A reference strains**

HIV Isolate	Viral Subtype	Tiered Category	Reference
MW965.25	C	1A	<i>NIH ARRRP#</i>
ZM197 M.PB7	C	1B	(Li et al., 2006)
ConC	C	2	(Kothe et al., 2006)
DU156.12	C	2	(Li et al., 2006)
DU172.17	C	2	(Li et al., 2006)
ZM214 M.PL15	C	2	(Li et al., 2006)
CAP45.G3	C	2	(Li et al., 2006)
CAP239.G3	C	2	(Gray et al., 2007)
SF162.LS	B	1A	(Stamatatos and Cheng-Mayer, 1998)
6535.3	B	1B	(Li et al., 2005)
AC10.0.29	B	2	(Li et al., 2005)
WITO 4160.33	B	2	(Li et al., 2005)
TRO.11	B	2	(Li et al., 2005)
QH0692.42	B	2	(Li et al., 2005)
PVO.4	B	3	(Li et al., 2005)
Q23ENV17	A	2	(Blish et al., 2007)
Q842ENVd12	A	2	(Blish et al., 2007)
Q168ENVa2	A	2	(Blish et al., 2007)
Q461ENVe2	A	2	(Blish et al., 2007)
Q769ENVd22	A	2	(Blish et al., 2007)

NIH ARRRP# - National Institutes of Health AIDS Research and Reference Reagent Program

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**Table 3**  
**Effects of single point mutations on neutralization sensitivity and summary of antibody specificities**

Plasma sample identity	ConC N160A			ConC K169E			Fold effect of mutation <sup>a</sup>			Antibody specificity conferring breadth			
	ConC N160A	ConC I165A	ConC K169E	CAP45 N160A	CAP45 I165A	CAP45 K169E	CAP45 G3 K169E	Du156.12 N332A	Q23.17 N332A	Tro.11 N332A	Q23.17 N332A	Unknown	Quaternary, PG9/PG16 like, N332
SK200	1.1	0.5	0.7	1.0	1.9	1.2	1.2	1.5	0	0	Unknown		
study exit SK221	0.4	0.3	1.1	2.0	5.6	1.4	1.4	0.6	4.2	4.2	Quaternary, PG9/PG16 like, N332		
study exit SK233 study exit	0.1	0	0.6	0.8	4	0.7	0.7	1.2	0	0	Quaternary, PG9/PG16 like		

<sup>a</sup> Calculated as wild type IC<sub>50</sub>/mutant IC<sub>50</sub> for the plasma. Changes in titer of >3 fold are shown in bold.