Envelope characteristics in individuals who developed neutralizing antibodies targeting different epitopes in HIV-1 subtype C infection

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Abstract

Broadly neutralizing antibodies (bNAbs) may constitute an essential component of a protective vaccine against HIV-1, yet no immunogen has been able to elicit them. To characterize the development of bNAbs in HIV-1 subtype C infected individuals, a panel of 18 Env-pseudotyped viruses was used to screen 18 study participants. The specificity of plasma neutralization was mapped against Env mutants and MPER chimeras. Envelope (env) gene sequence evolution was characterized by single genome amplification and sequencing. Three out of eighteen individuals developed broad plasma neutralizing activity (>60% breadth). Two of the three participants may target epitopes comprising glycans at position 276 of the D loop in the CD4 binding site and 332 glycan supersite, respectively. Deletion of these glycans was associated with neutralization resistance. Our study describes the kinetics of the development of plasma neutralizing activity and identified amino acid residue changes suggestive of immune pressure on putative epitopes. The study enhances our understanding of how neutralization breadth develops in the course of HIV-1 subtype C infection.

Keywords: Broadly neutralizing antibodies; Envelope sequencing; Epitope mapping

1 Background

In 2018, there were 1.7 million new cases of human immunodeficiency virus type 1 (HIV-1) infection worldwide, and half of these occurred in Eastern and Southern Africa (UNAIDS, 2019). These data underscore that current HIV-1 prevention strategies are suboptimal and highlight the need for an effective preventative HIV-1 vaccine that would be expected to have the most significant and durable impact in reducing transmission worldwide. A successful preventative HIV-1 vaccine will need to overcome the extreme genetic diversity of the strains that comprise the HIV epidemic globally and ideally induce broadly neutralizing antibodies (Mann and Ndungu, 2015). Passive administration of broadly neutralizing monoclonal antibodies (bNAbs) has been shown to prevent infection or delay viral rebound in non-human primate animal models of HIV-1 infection (Nishimura et al., 2017; Schoofs et al., 2016; Julg et al., 2017; Baba et al., 2000; Hessell et al., 2009a, 2009b). Recently, these antibodies have also been shown to be safe and effective in delaying viral rebound in humans chronically infected with HIV-1, raising the possibility of their ultimate application in both therapeutic and preventative strategies against HIV-1 (Scheid et al., 2016; Caskey et al., 2017; Gautam et al., 2018; Wagh et al., 2018a; Bar-On et al., 2018). The CD4 binding site monoclonal antibody (mAb) VRC01 is currently in a phase 2b human clinical trial to assess if passive administration is effective in preventing HIV-1 infection in high risk populations (Ledgerwood et al., 2015). However, despite the promise that bNAbs hold for HIV prevention and therapy,
passive administration is expensive and unlikely to be a feasible public health intervention. Significant challenges therefore remain because no vaccine immunogens have been able to induce such antibodies and the mechanisms that lead to the development of these antibodies in natural infection are not fully understood.

During infection, almost all HIV-1 infected individuals develop strain-specific neutralizing antibodies within 3–12 months of infection (Wei et al., 2003; Richman et al., 2003). However, HIV-1 employs multiple strategies to escape from the immune pressure exerted by these antibodies (Wei et al., 2003; Richman et al., 2003; Rong et al., 2009; (Moore et al., 2009). In contrast, bNAbs develop in only 10–30% of HIV-1 infected individuals from approximately 2-3 years post-infection, but do not improve clinical outcome (Li et al., 2009; Doria-Rose et al., 2009; Stamatatos et al., 2009; van Gils et al., 2009; Gray et al., 2011a; Sather et al., 2009; Hraber et al., 2014; Landais et al., 2016). A large body of evidence suggests that the development of bNAbs is associated with the duration of infection, higher viral load, lower CD4+ T cell count and a higher frequency of T follicular helper cells (van Gils et al., 2009; Gray et al., 2011a; Sather et al., 2009; Landais et al., 2016; Derdeyn et al., 2014; Euler et al., 2010; Locci et al., 2013; Cohen et al., 2014). A recent study reported associations between the development of bNAbs and subtype C infection, HLA-A*03 genotype and higher anti-Env IgG binding antibody titers (Landais et al., 2016). Another study reported that HIV-specific antibody-dependent complement deposition (ADCD) and cellular trogocytosis (ADCT) predict the development of bNAbs suggesting that these immune functions in the germinal center provide a link between Fc and Fab region of the antibodies (Richardson et al., 2018). Envelope (env) gene sequencing from plasma of individuals that developed bNAbs suggested that targeted viral evolution and higher viral load are important for the development of bNAbs (Piantadosi et al., 2009, Powell et al., 2010); Cortez et al., 2012; Rusert et al., 2016; (Mabvakure et al., 2019). However, it is still not clear why some individuals develop bNAbs while others do not. Therefore, studies that investigate the factors associated with the development of bNAbs in infection may be important for rational design of strategies to induce such antibodies through vaccination.

Technologies including antigen-specific B cell sorting and B cell cultures with micro-neutralization assays have led to the isolation of more than 100 monoclonal antibodies with remarkable neutralization breadth and potency (Tiller et al., 2008, Wu et al., 2010). These monoclonal antibodies have been shown to target six epitopes on the viral Env glycoprotein; the CD4 binding site (CD4bs), gp41 membrane-proximal external region (MPER), gp41 fusion peptide, the gp120-gp41 interface, V2-glycan, and the V3/C3 site on gp120 (West et al., 2014; Kong et al., 2016). However, some plasma samples with cross-neutralizing activity do not map to any of the known epitopes (Wibmer et al., 2013; Ditse et al., 2018) suggesting that there may be other important specificities yet to be characterized. Therefore, identification of chronically HIV-1 infected participants with breadth may reveal new epitopes on the HIV-1 Env that may be targeted in vaccine development and help to unravel the viral evolutionary pathways or early events that lead to the emergence of these antibodies.

Several studies have characterized the co-evolution of HIV-1 Env and neutralizing antibodies in the host and identified multiple pathways of bNAb development (Wibmer et al., 2013; Liao et al., 2013a; Doria-Rose et al., 2014; (Moore et al., 2012); Bonsignori et al., 2017; Kouyos et al., 2018). Some studies indicate that viral escape from strain-specific neutralizing antibodies may create or expose a conserved epitope, which may include the insertion of a vulnerable glycan leading to the development of bNAbs (Moore et al., 2012, Wibmer et al., 2013).
Other studies suggest that repeated cycles of sequential viral escape from strain-specific neutralizing antibodies drive somatic mutation and affinity maturation of the antibody leading to tolerance of epitope variation and the development of bNAbS (Liao et al., 2013a; Doria-Rose et al., 2014, 2015; (Moore et al., 2012; Bhiman et al., 2015; Bonsignori et al., 2016; Gao et al., 2014; MacLeod et al., 2016). Thus, characterization of specific envelope signatures in HIV-1 subtype C infected individuals with bNAbS may also be important in understanding subtype-specific pathways for bNAb development and could inform vaccine design (Kouyos et al., 2018).

In this study, we first screened eighteen individuals with HIV-1 subtype C infection who were longitudinally followed from acute infection. We then characterized the kinetics of development of bNAbS in three individuals. In addition, we mapped the epitope specificities of broadly neutralizing antibodies and sequenced the virus longitudinally to assess the evolution of HIV-1 Env. Three individuals developed bNAbS at approximately 18 months post-infection. One individual developed bNAbS that may target the epitope containing glycans at position 276 glycan on loop D of the CD4 binding site and these neutralizing antibodies had the same specificity over time. In the second individual bNAbS may target an epitope that contains the N332 glycan on the base of the V3 loop and the specificity did not change over time. In these two individuals, neutralization escape was associated with the deletion of glycans on previously identified HIV-1 Env epitopes. In the third individual, antibody specificity did not map to any of the known epitopes suggesting that bNAbS might be targeting known epitopes but with subtle differences compared to known bNAbS. Analysis of the Env sequences indicated that bNAbS may target a known epitope but with diverse fine specificity mediated by amino acids within or adjacent to an epitope. Alternatively, neutralization within an individual may also be mediated by antibodies targeting different epitopes.

2 Materials and methods

2.1 Study participants

Serum samples were obtained from eighteen antiretroviral treatment-naïve individuals with known timing of HIV-1 subtype C infection. These participants had at least 4 years of longitudinal follow up in the HIV Pathogenesis Programme (HPP) Acute Infection study in Durban, KwaZulu-Natal, South Africa (Bassett et al., 2011). The study was approved by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal and the Institutional Review Board of Massachusetts General Hospital. The participants provided written informed consent prior to participating in the study.

2.2 Viral load and CD4+ T cell count

Plasma HIV-1 RNA was measured using the Amplicor ver 1.5 assay (Roche Molecular Systems, Branchburg, NJ, USA). Meanwhile, CD4+ T cells were counted using Tru-count technology and analyzed further with flow cytometry according to manufacturer's instructions (Becton Dickinson, BD Biosciences, San Jose, CA, USA).

2.3 Cell lines
The CD4+ CCR5+ TZM-bl cell line was obtained from NIH AIDS Research Center (NIH, AIDS Reagent Program, Bethesda, MD, USA), while the 293T cell line was obtained from the American Type Culture Collection (ATCC, Manassas, CA, USA). The cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) with 10% heat-inactivated fetal bovine serum (Gibco BRL Life Technologies), 25 mM HEPES (Life Technologies, Taiwan) and 50 µg/ml gentamicin (Sigma Aldrich, St Louis, MD, USA). The cells were incubated at 37 °C, 5% CO₂ and the medium was replaced every two days. The medium was decanted and monolayers were rinsed with sterile phosphate buffered saline (PBS) and trypsinized with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Invitrogen, Grand Island, NY, USA). The cells were re-suspended in growth medium and counted manually using trypan blue and a hemocytometer.

2.4 Pseudovirus production

Previously, studies used a panel of diverse HIV-1 positive serum to assess the sensitivity of virus panels and ranked the viruses according to their neutralization sensitivity (Seaman et al., 2010). Four groups were identified, highly sensitive viruses were classified into tier 1A, above-average were tier 1B, while moderate sensitivity were tier 2 and resistant viruses were classified into tier 3 (Seaman et al., 2010). Eighteen env plasmid deoxyribonucleic acid (DNA) clones representing heterologous Env from a panel of 6 HIV-1 subtype A, 6 B, and 6 C tier 2 and 3 viruses were co-transfected with an Env-defective backbone plasmid (pSG3Δenv) (obtained from the NIH AIDS Research and Reference Reagent Program) into 293T cells (supp table 1) (Gray et al., 2011a; Wibmer et al., 2016). Co-transfection was mediated by the X-tremeGene 9 DNA transfection reagent and the cultures were incubated for 48 h at 37 °C (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's instructions. Pseudoviruses were filtered through the 0.45 μm filter and frozen.

2.5 Determination of infective dose (TCID₅₀)

Infective dose (TCID₅₀) was assessed by preparing four-fold dilutions of the pseudovirus in a 96 well plate. TZM-bl cells containing dextran-hydrochloride (DEAE) were added to the virus and TZM-bl cells not treated with the virus were used as a negative control. The plate was incubated at 37 °C, 5% CO₂ for 48 h. After incubation, growth media was removed and replaced with luciferase reagent and viral infectivity was measured as relative light units (RLUs) using a luminometer. A cut-off value of 2.5 times higher RLUs was used to quantify positive infection. The infective dose that yielded ≤50 000 RLUs was used for the neutralization assays.

2.6 Neutralization assays

Neutralization assays against heterologous HIV-1 pseudoviruses were performed as described previously (Gray et al., 2011a). Heat inactivated plasma samples were incubated with the single-round competent pseudoviruses. TZM-bl cells containing DEAE (Sigma-Aldrich, St Louis, MO, USA) were added in a 96 well plate and incubated for 48 h at 37 °C. Tat-regulated firefly luciferase (Luc) reporter gene expression was used to quantify reductions in virus infection in TZM-bl cells. Neutralization titers were measured on a luminometer and reported as Relative Luminescence Units (RLUs) (PerkinElmer Life Sciences, Separation Scientific,
Model Victor 2). Geometric mean titers (GMT) were estimated by calculating the average serum dilution required to neutralize 50% (ID_{50}) of all the viruses tested. TZM-bl cells not exposed to the virus were used as a negative control, while the pseudotyped viruses and the TZM-bl cells were used as a positive control. Vesicular stomatitis virus G (VSVG) pseudotyped virus incubated with TZM-bl cells was used to rule out non-specific inhibition. All neutralization experiments were done at least twice.

2.7 Mapping of HIV-1 epitopes

The specificity of bNAbss was determined by testing neutralization capacity of participant plasma at 3 years post infection against Env single amino acid mutants. Mutant env genes were generated by site-directed mutagenesis using the Stratagene Quick-change II kit and co-transfected with pSG3Δenv HIV-1 backbone on 293T cells (Agilent Technologies, Santa Clara, CA, USA) as previously described (Moore et al., 2012). Neutralization assays in TZM-bl cells were performed and compared between wild type and mutant viruses. A threefold reduction in neutralization of a mutant compared to wild type pseudovirus was considered a potential target of the broadly neutralizing antibodies. Membrane proximal external region (MPER)- specific antibodies were assessed by performing neutralization using HIV-1 C1 chimera and compared to HIV-2 7312A chimera as previously described (Gray et al., 2009). Mapping for fusion peptide antibodies was done by enzyme-linked immunosorbent assay (ELISA), 96-well streptavidin coated plates were coated with biotinylated fusion peptide and incubated at 37 \degree C for 2 h. The fusion peptide sequence was obtained from participant AS2-0358. The plates were blocked with bovine serum albumin (BSA) and washed with PBS-1% Tween. Serially diluted plasma was added into the plate and incubated at 37 \degree C for 1 h. Non-specific antibodies were washed with PBS-1% Tween and the plate was incubated with goat anti-human Fcγ IgG conjugate with horseradish peroxidase (HRP) at 37 \degree C for an hour. After incubation, the plate was washed 6 times with PBS-Tween and were developed with 3, 3',5, 5'-tetramethylbenzidine (TMB) and read at 450 nm.

2.8 Neutralization fingerprinting

Neutralization fingerprinting analysis was performed as previously described (Raju et al., 2019). Briefly, the HIV-1 neutralization panel for the fingerprinting analysis included 18 diverse HIV-1 strains against which the polyclonal sera were tested; two strains (BG505 and CAP239.G3) were excluded since there was insufficient monoclonal antibody data against these strains, resulting in a total of 16 strains for the fingerprinting analysis. Monoclonal antibody neutralization data for the selected strains were retrieved for a set of broadly neutralizing antibodies, grouped into the following representative clusters: VRC01-like (VRC01, VRC-PG04, VRC-CH31, 3BNC117, VRC-PG20, VRC23, VRC27), HJ16-like (HJ16), 8ANC195-like (8ANC195), PG9-like (PG9, PGT145, CH01), PGT128-like (PGT121, PGT128), 2F5-like (2F5), 10E8-like (10e8, 4E10), PGT151-like (PGT151), VRC34-like (N123-VRC34.01) and VRC40-like (CH540-VRC40.01). The overall prevalence of each of these antibody specificities in each of the sera was predicted using the neutralization fingerprinting algorithm (Raju et al., 2019).

2.9 Single genome amplification and sequencing

For single genome amplification (SGA) of the full-length env gene, a limiting endpoint dilution was performed on complementary deoxyribonucleic acid (cDNA) as previously described (Salazar-Gonzalez et al.,
Briefly, cDNA was serially diluted in replicates of eight polymerase chain reaction (PCR) wells and nested env PCR was performed. cDNA dilutions were tested to identify a dilution where ~30% of wells were positive for amplification products. Overlapping DNA fragments were assembled and edited using Sequencher Program v8.1.2 (Gene Codes Corporation, Michigan, USA) and aligned using ClustalW. Maximum-likelihood phylogenetic trees were constructed as implemented by Geneious v.8.1.6 to evaluate clustering of sequences with each other as well as with subtype reference strains obtained from the Los Alamos HIV sequence database.

2.10 Statistical analysis

Statistical comparisons were performed in GraphPad Prism 5.0 (GraphPad Prism version 5.0 for windows, GraphPad Software, San Diego California, USA). Statistical significance was observed where p-values were ≤0.05. Correlations of the percentage of viruses neutralized with viral load or CD4+ T cell counts were measured using Spearman nonparametric test. Differences in amino acid lengths and N-linked glycosylation sites (PNGS) in the V1 region at 6, -30 and -136 weeks post-infection were assessed using analysis of variance (ANOVA) and Bonferroni adjustments.

3 Results

3.1 Broadly neutralizing antibodies developed at approximately 1.5 years post infection in 3 HIV-1 subtype C infected participants

Our first goal was to identify HIV-1 subtype C infected individuals who developed broadly neutralizing antibodies, indicated by neutralization of more than 40% of heterologous pseudoviruses (Gray et al., 2011b). Eighteen HIV-1 infected individuals with known timing of infection were screened for bNAb at 3 years post-infection using a panel of 18 heterologous subtype A, B and C pseudoviruses. Three out of eighteen (17%) individuals (AS3-0268, AS2-1037 and AS2-0358) neutralized more than 60% of heterologous viruses and were classified as broad neutralizers. In contrast, plasma from the other fifteen individuals only neutralized 0–22% of heterologous viruses tested (Fig. 1). Plasma sample from AS3-0268 neutralized some viruses particularly potently (Du15.12, CAP45 G3, TRO.11, Q42.d12 and BG505.N332+) (GMT titer of 285) and no subtype preference was observed (Fig. 1). Participant AS2-1037 neutralized 78% of heterologous viruses tested with moderate potency (GMT titer of 123).
AS2-1037 plasma antibodies neutralized 6/6 of subtype C, 5/6 of subtype B viruses, but only 3/6 of subtype A viruses. Last in this group of broad neutralizers was participant AS2-0358 whose plasma neutralized 61% of viruses tested with a moderate potency (GMT titer of 99) (Fig. 1).

3.2 Broad neutralization activity increased in breadth and potency over time

To determine the kinetics of the development of bNAbs, we characterized longitudinal plasma samples starting at approximately 4 weeks (1 month) of infection to approximately 200 weeks (4 years) post-infection. All three individuals showed limited neutralization of heterologous pseudoviruses during the first year of infection. Participant AS3-0268 developed heterologous neutralization at approximately 1.5 years (75 weeks) post-infection. Neutralization breadth and potency increased over time and reached a peak at 3 years (150 weeks) post-infection. This participant neutralized 89% (16/18) of pseudoviruses, with neutralization titers decreasing slightly from 150 to 200 weeks post-infection (p = 0.06; paired t-test) (Fig. 2a) Neutralization responses were dominated by subtype A and C viruses and neutralization activity was low against subtype B viruses. Interestingly, plasma from participant AS3-0268 did not neutralize WITO04160 and CAP239 viruses. At ~4 years (200 weeks) post infection, AS3-0268 plasma maintained neutralization breadth for 44% of the viruses including CAP45.G3, Q842.d12, BG505 N332+, Du156.12, Du172.17, TRO.11, AC10.0.29, QH692d12 and Q167.a2 at slightly lower titers suggesting neutralization escape. However, neutralization titers increased for 39% of viruses including Q23.17, Q769.d22, 6535.3, ZM197.PB7, ZM214.PL15, PVO.4, and Q461.e2 (Fig. 2a).
Kinetics of neutralizing antibody breadth in three patients: AS3-0268 (a), AS2-1037 (b) and AS2-0358 (c) using the standard panel of 18 (6 subtype A, 6 B and 6 C) viruses. The graph shows ID50 values against tested viruses over time following HIV-1 infection. Viruses are colour-coded by subtype, pink lines indicate subtype C, green lines indicate subtype B and blue lines indicate subtype A viruses. For participant AS2-1037, samples were not available between 30 and 83 weeks post-infection. The dotted line indicates the time point of plasma neutralization epitope mapping using site-directed mutagenesis.

In contrast, in participant AS2-1037 bNABs were observed at approximately 1.5 years (83 weeks) post infection and neutralized 61% (12/18) of heterologous viruses. However, the exact timing of development of bNABs could not be determined in this participant due to sample unavailability between 30 and 83 weeks post-infection. Neutralization breadth increased over time, such that plasma at 97 weeks post infection neutralized 78% (14/18) of heterologous viruses, with a slight decline in both neutralization breadth and
potency thereafter (p = 0.10) (Fig. 2b). Interestingly, neutralization titers increased again between weeks 150 and 175 post infection and there was no subtype preference. Participant AS2-0358 developed bNAbs at nearly 1.5 years (75 weeks) post infection, neutralizing 44% (8/18) of heterologous viruses. Neutralization breadth increased gradually over the follow up period, reaching a plateau at approximately 2.5 years (125 weeks) post- infection. At approximately 3 years (162 weeks) post infection, plasma from AS2-0358 neutralized 61% (11/18) of the heterologous viruses tested, with neutralization breadth of 4/6 against both subtype C and A, while it neutralized 3/6 against subtype B (Fig. 2c). Interestingly, neutralization activity in this participant increased slightly from 162 to 200 weeks post-infection and there was no subtype preference.

### 3.3 Associations of bNAbs with markers of disease progression

Higher viral load and lower CD4+ T-cell counts have previously been associated with the development of neutralization breadth in chronic HIV-1 infection (van Gils et al., 2009; Gray et al., 2011a; Landais et al., 2016; Euler et al., 2010). To evaluate the factors associated with the development of bNAbs in our cohort, we evaluated the correlations between neutralization breadth, viral set point (average viral load from 3 to 12 months post-infection) viral loads and CD4+ T-cell counts at 1 month, 6 months and 12 months post-infection. There was a borderline positive correlation between neutralization breadth and viral load at 1 month (p = 0.0616; r = 0.5316), but this correlation reached significance at 6 months (p = 0.0315; r = 0.5558) and 12 months post-infection (p = 0.0020; r = 0.7312) (Fig. 3a-c). However, the correlations were rather weak and appeared dependent on the three individuals that developed breadth. In contrast, there was no correlation between neutralization breadth and CD4+ T cell counts at any time point (Fig. 3d-f). Nevertheless, the three participants that developed neutralization breadth had the lowest CD4+ T cell counts at 1 and 6 months post-infection (Fig. 3d and e). There was no correlation between percentage of viruses neutralized and viral set point or the rate of CD4+ T cell decline (data not shown).
3.4 Plasma neutralizing antibodies from patient AS3-0268 and AS2-1037 may target epitopes that are influenced by the N-linked glycans at position 276 and 332 of the gp120 subunit respectively

We next assessed epitope specificities of bNAbs in the three participants that developed neutralization breadth (Gray et al., 2009). Single point mutations were introduced in the CD4 binding site (N276A and N234S), V2 apex (N160K, K169E) and C3/V3 loop (N332A) and gp120-gp41 interface (N88A, N637A, N611D) of either CAP45.G3, Du156.12 or TRO.11. Monoclonal antibodies HJ16, PGT121 and VRC01 were used as positive controls for mapping (Table 1). The presence of MPER-specific antibodies in plasma was assessed using HIV-2/HIV-1 MPER chimeras as previously described (Gray et al., 2009), whereas the presence of fusion peptide-specific antibodies was determined by ELISA using the fusion peptide sequence derived from AS2-0358 participant. Our data showed no MPER-specific antibodies in plasma as there was no difference in neutralization of C1 MPER and 7312A chimeras (Supp Fig 2). However, AS3-0268 plasma neutralization activity was reduced 12-fold when an N276A mutation was introduced in CAP45.G3 and 4-fold reduction when this mutation was inserted into TRO.11 (Table 1, Fig Supp 1A). Neutralization fingerprinting was consistent with our findings; specifically, the AS3-0268 plasma was predicted to have VRC40-like antibodies which target an epitope containing the N276 glycan (Table 2). We also found weak fusion peptide responses by ELISA however, these may be the binding antibody responses (Supp Fig 2b). These findings suggest that bNAbs in this participant may target an epitope comprising or influenced by the N-linked glycan at position 276. However, it should be noted that there was only partial reduction in neutralization sensitivity of the N276A variant in the TRO.11 backbone, which suggests that this participant may have antibodies.
The dependence of AS3-0268 neutralization on N276 and AS2-1037 on N332 glycans compared to V2 domain and gp120-gp41 interface. Average fold differences between wild type CAP45.G3, TRO.11, Du156.12 and Gpi20 mutants (N160K, K169E, N276A, N234A, N331A, N88A, N637A and N611D) calculated from 3 independent experiments. Monoclonal antibodies: PGT121, HJ16 and PG9 were used as positive controls. Fold reductions >3 are highlighted in red, while 0-3-fold reductions have clear squares. A dot (.) indicates missing neutralization fold changes due to the unavailability of the mutant.

Neutralization fingerprinting analysis for participants AS3-268, AS2-1037 and AS2-358 at approximately 3 years post-infection.

AS2-1037 plasma neutralization showed a five-fold reduction in neutralization when tested against two different viruses bearing the N332A mutation (Table 1, supp Fig 1b). Neutralization fingerprinting also indicated that AS2-1037 plasma have PGT128-like antibodies that target the N332-glycan supersite (Table 2). These findings indicate that antibodies in this participant may target an epitope comprising N-linked glycan at position 332 of the C3/V3 region of gp120. Longitudinal epitope mapping using TRO.11 mutants indicated that neutralizing antibodies have the same specificity that slightly increased in potency from 83 to 175 weeks targeting other regions of the envelope (Supp Fig 1A; Supp Table 2). N276-glycan specific antibodies (HJ16 and 8ANC131) target a subsite of the CD4 binding site, they are derived from VH1-46 gene and they have a long CDR-H3 loop which is crucial for their binding (Corti et al., 2010; Liao et al., 2013b). Unlike VRC01-like antibodies, HJ16 and 8ANC131 antibodies do not interact with D368 residue. They depend entirely on a glycan at position N276 (Balla-Jhaghoorsingh et al., 2013). Interestingly, some VRC01-like antibodies bind to the N276 glycan, however it is not critical for their neutralization.
post-infection (Supp Table 2) (p > 0.05). Lastly, for participant AS2-0358 there was no significant effect on neutralization sensitivity for mutants directed at the V2 glycan, CD4 binding site, C3/V3, MPER and gp120- gp41 interface. However weak binding antibody responses to the fusion peptide were detected at 3 years post- infection (Supp Fig 2b). Neutralization fingerprinting also predicted that this participant may have antibodies against the fusion peptide, as indicated by VRC34-like antibody responses (Table 2). These findings suggest that bNAbs in this participant may target the fusion peptide or other epitopes that are not glycan-dependent.

3.5 N-linked glycans are conserved during the first year of infection and they are deleted as a result of neutralization pressure

To investigate viral evolutionary pathways during the course of infection, we performed SGA followed by sequencing of the envelope. Our epitope mapping data suggested that plasma from AS3-0268 may target an epitope that contains N-linked glycan at position 276 on loop D of gp120. Single genome amplification and sequencing of the envelope was performed at approximately 4, 36 and 150 weeks post-infection. We analyzed previously identified CD4 binding site amino acid positions in gp120 (N234, N276, N278, N279), the D368 residue critical for VRC01-like antibodies (D368, E370), the D474 residue critical for HJ16-like antibodies (D474, M475) and the base of V5 region (R456) (Wibmer et al., 2013; Li et al., 2011). Analysis of loop D on AS3-0268 showed that N-linked glycans were conserved at position 276 during the early stages of infection (4 to 36 weeks post-infection). However, the glycan was deleted through a N276S mutation in 67% (8/12) of the viral variants by 150 weeks post-infection (Fig. 4a). These presumed escape variants coincided with a slight reduction in neutralization titers from 150 to 200 weeks post-infection (p = 0.06), although the exact timing of emergence of mutations is not known (Fig. 2a). In addition, at 150 weeks post-infection, one viral variant mutated from asparagine (N) to aspartic acid (D) at position 279 and this is a common mutation, that has been previously described (Wibmer et al., 2013).

Viral sequence characterization of potential antibody specificities and quasispecies in acutely infected participants AS3-268, AS2-1037 and AS2-358. Longitudinal single genome sequences from AS3-268 autologous viruses at 6 weeks, 36 weeks and
Our findings indicated that participant AS2-1037 may target an epitope that contains N-linked glycan at position 332 of the V3 loop. Analysis of the V3 region showed that the N-linked glycan at position 301 and 332 was conserved, however several other changes were observed in the V3 region from 6 to 30 weeks post-infection (Fig. 2b). During this period of infection, heterologous neutralization increased in breadth and potency (Fig. 2b). The N332 glycan was lost in 29% (5/17) of viral variants at approximately 136 weeks post-infection and one of the five viral variants gained a glycan (N) at position 334 (Fig. 4b). Mutations in position 332 coincided with reduced neutralization potency from 100 to 150 weeks post infection (Fig. 2b). In addition, this participant had serine (S) in position 334 from 6 to 30 weeks p.i (Fig. 4b) which was replaced by asparagine (N) at 136 weeks post-infection in 81% (13/17) of viral variants. Interestingly, neutralization titers increased again from 150 to 160 weeks post infection suggesting that appearance of a glycan in position N334 might have improved the neutralization activity.

For participant AS2-0358, we were unable to map the specificity of broadly neutralizing antibodies. However, sequence analysis revealed immune pressure that could be indicative of bNAb specificities. At 4- and 32 weeks post infection, V2 epitope N-linked glycans at N156 and N160 were conserved (Fig. 4c). However, the N-linked glycan was replaced with tyrosine (Y) at position 160, while lysine (K) was replaced with arginine (R) at position 166 by 162 weeks post-infection (Fig. 4c). In addition, alanine (A) was replaced with glutamic acid (E) at position 172 in 19/19 of viral variants. Both the N160 glycan and K166 are highly conserved, therefore these mutations are suggestive of immune pressure on the V2 epitope. To our knowledge, the N160Y, K166R and A172E mutations have not previously been reported as indicative pressure on this epitope and will therefore require additional confirmatory studies.

Analysis of the D-loop and the base of V5 indicated that viral variants at 4 to 32 weeks possessed asparagine (N) at position 279 and an unusual serine (S) at position 456 (Fig. 4d and e). However, by approximately 162 weeks post-infection, the asparagine (N) at position 279 had mutated into aspartic acid (D) in 14/19 of viral variants (Fig. 4d). In addition, lysine (K) had been replaced by glutamic acid (E) at position 275, while serine (R) was replaced with asparagine (N) in position 280, and valine (V) was completely replaced with alanine (A) in all viral variants at position 281 (Fig. 4d). We analyzed the envelope sequences from reference strains including HXB2, ancestral subtype C, consensus subtype C and CAP45 and found that all these possessed glutamic acid (E) at position 275, aspartic acid (D) at position 279 and alanine (A) at position 281. These findings suggest that the mutations observed in participant AS2-0358 may also indicate an important region that may be targeted by these antibodies.

Analysis of the V5 domain indicated that serine (S) reverted to arginine (R) at position 456 which is the most common global residue (Fig. 4e). The length of V5 region shortened from 4 to 32 weeks and then elongated from 32 to 162 weeks post infection (supp Fig 3). Most of the changes on the D-loop and V5 domain were reversions into common residues and likely did not indicate bNAb pressure. The changes in viral Env
sequence suggested that there was an immune escape within the V2 loop. In addition, the fusion peptide sequence was highly conserved (data not shown), however mapping experiments showed weak FP responses. Taken together, these findings suggest that AS2-0358 plasma neutralization capacity may be due to the additional effects of several targeted epitopes.

3.6 Long variable region 1 during acute HIV-1 infection in the participant who developed N332-specific antibodies

The impact of an infecting virus on the development of neutralization breadth is thought to be moderate, however it allows the identification of viral strains that induce the development of bNAbs and this is crucial in vaccine development studies (Kouyos et al., 2018). Studies have shown that a longer and highly glycosylated V1 domain shields HIV-1 and prevent recognition by V3-glycan directed bNAbs (van den Kerkhof et al., 2016; Silver et al., 2019). We next assessed amino acid length and number of potential N-linked glycosylation (PNGS) sites on the V1 loop. Interestingly, participant AS2-1037 was infected with a virus that had a very long V1 domain (40 amino acids), with only two variants possessing short (19 AAs) V1 domains (Fig. 5). However, the V1 domain had mutated to a significantly shorter version (19–26 AAs) in all viral variants at 30 and 136 weeks post-infection. In addition, viral variants at 6 weeks post-infection also had more N-linked glycosylation sites within the V1 domain compared to viruses at 30 and 136 weeks post-infection (Fig. 5). These findings suggest that AS2-1037 was infected with a virus from a donor that previously escaped from V3-specific antibodies as the longer elongation of V1 domain was previously associated with neutralization escape (van den Kerkhof et al., 2016; Silver et al., 2019; Walker et al., 2011a).

4 Discussion

In this study, we screened for neutralization breadth in eighteen individuals longitudinally followed from acute HIV-1 subtype C infection, characterized the kinetics of the development of neutralization breadth over time and mapped neutralization specificities using single point mutants. In addition, we also analyzed the
associations between markers of disease progression and the development of bNAbs. Finally, we longitudinally analyzed the env genes of subjects that developed breadth, using single genome amplification and sequencing within the known bNAb epitopes on neutralization activity over time.

Overall, three individuals (17%) developed bNAbs by approximately 1.5 years post infection, showing neutralization against a majority of tier 2 virus panel including subtype A, B and C. This prevalence is consistent with data reported from previous studies that used larger cohorts of HIV-1 infected participants (van Gils et al., 2009; Gray et al., 2011a; Landais et al., 2016). Longitudinal characterization of heterologous neutralization showed that bNAbs develop at approximately 1.5 years post-infection, neutralization breadth and potency increased over time. Previous studies reported that the development of bNAbs is associated with higher viral load and lower CD4+ T cell counts (van Gils et al., 2009; Gray et al., 2011a; Landais et al., 2016; Rusert et al., 2016). In this study, higher viral load was also associated with the development of bNAbs, although the correlation is weak and only three participants developed breadth.

Previous studies have shown that HIV-1 antibody target sites tend to be covered by glycans and therefore they are not easily accessible to neutralizing antibodies, however, most bNAbs have evolved to target N-linked glycans that shield the conserved sites on the envelope (Gray et al., 2009; Walker et al., 2009, 2011b; Tomaras et al., 2011). Participant AS3-0268 developed broad and potent bNAbs responses that neutralized 89% of viruses tested and targeted the epitope that comprised N-linked glycans at position 276 in the D loop of gp120. Longitudinal mapping indicated a consistent specificity that increased in potency over time. Analysis of single genome amplification sequences indicated conserved N-linked glycosylation sites during the early stages of infection. However, the deletion of glycans and substitution with serine (N276S) and substitution of asparagine with aspartic acid (N279D) at 150 weeks post infection might have led to neutralizing escape. These findings strongly suggest that this participant developed bNAbs targeting an epitope comprising N276-glycan within the CD4 binding site. However, the plasma antibodies in this participant may also target other specificities. Previous studies reported that substitution of N-linked glycosylation sites with alanine at position 276 leads to resistance to neutralization mediated by the N276-specific antibodies that do not interact with D368 particularly the HJ16 mAb (Balla-Jhagjhoorsingh et al., 2013; Lavine et al., 2012). In contrast, these studies found increased sensitivity of N276A mutated viruses to the CD4 binding site antibodies that interact with D368 including VRC01 and VRC03 (Balla-Jhagjhoorsingh et al., 2013; Li et al., 2011). AS3-0268 plasma neutralization activity was reduced when tested against N276A mutants suggesting that this participant may have developed HJ16-like antibodies. There are only a few of isolated N276-specific monoclonal antibodies including HJ16, 179NC75 and CAP257-RH1 (Wibmer et al., 2013, 2016; Corti et al., 2010; Balla-Jhagjhoorsingh et al., 2013; Freund et al., 2015). This study identified an HIV-1 subtype C infected individual that developed potent bNAbs targeting an epitope that contains N-linked glycans at position 276 and this participant may provide broad and potent monoclonal antibodies that could contribute to further characterization of the humoral immune response against HIV-1 and vaccine development.

Previously, our group showed that this participant was infected with four different viral variants (Gounder et al., 2015). HIV-1 gag was sequenced at 34 days post infection and showed a heterogenous population with 4 different viral variants. Our analysis therefore suggests that infection with multiple viral variants may have led
to the development of bNAbs in this participant. It is possible that infection with multiple variants may have caused a diverse antibody response and primed for the development of bNAbs. This would be in contrast to superinfection, whereby a new variant sharing epitopes with an established virus does not necessarily drive the development of bNAbs (Sheward et al., 2018). The impact of the characteristics of the transmitted founder viruses on development of neutralization breadth have been reported in several studies (van den Kerkhof et al., 2016; Wagh et al., 2018b; Smith et al., 2016). Longitudinal analysis of HIV-1 Env showed that transmitted/founder viruses with intact glycan shields and more efficient glycosylation were associated with the development of greater neutralization breadth (Wagh et al., 2018b; Smith et al., 2016).

A recent study introduced the concept of bNAb imprinting which involves identification and characterization of viruses that have the ability to induce cross-neutralizing antibodies for the development of immunogens (Kouyos et al., 2018). Env sequence analysis of AS2-1037 revealed that N-332 glycans were conserved in viral variants generated from 6 to 30 weeks post-infection. Nevertheless, the viral variants developed N332S escape mutations at approximately 162 weeks post-infection and this was accompanied by a slight decline in neutralization titers between 2 and 3 years post infection. Interestingly, all viral variants had serine at position 334 from 6 to 30 weeks post infection, which was replaced by asparagine (N) at 162 weeks post-infection, whereas neutralization titers increased from 150 to 160 weeks post-infection suggesting that this mutation might have improved neutralization in this participant. This suggests that the mutation from serine to asparagine might cause a subtle change in neutralization sensitivity through some yet undefined mechanisms since specificity did not change in mapping over time. In contrast, a previous study characterized two HIV-1 subtype C infected patients (CAP177 and CAP314) that targeted the N332-supersite (Moore et al., 2012). In both patients, the transmitted/founder virus did not have the N332 glycan, however it only developed at approximately 5 to 6 months post-infection due to viral escape from strain-specific neutralization.

Interestingly, analysis of the V1 domain indicated that AS2-1037 was infected with a transmitted/founder virus that had the longest V1 domain and increased PNGS which shortened by week 30 post-infection and this process coincided with the development of V3-specific bNAbs. These findings suggest that AS2-1037 was infected with a virus that previously escaped from V3-specific antibodies as described by others (van den Kerkhof et al., 2016). We plan to test whether the longer V1 has any impact on replication capacity of the virus as previously reported (van den Kerkhof et al., 2016; Silver et al., 2019). Our findings suggest that a longer V1 domain during acute infection might prevent the development of the N332 supersite specific antibodies, while shortening of the V1 domain exposes the N332 supersite and lead to the development of bNAbs.

In this study, we could not determine the specificity of antibodies from AS2-0358, however Env sequence analysis revealed that the viral variants from 0 to 32 weeks following infection had conserved V2, loop D and the base of V5 of the CD4 binding site. Interestingly, at 162 weeks post infection, we observed an unusual deletion of N-linked glycans and replacement with tyrosine (Y) at position 160, lysine (K) with arginine (R) at position 166 and alanine (A) with glutamic acid (E) at position 172 in all viral variants. These mutations suggested neutralization escape from immune pressure exerted by broadly neutralizing antibodies. We also noted mutations in loop D of gp120 including replacement of lysine with glutamate in position 275 (K275E), asparagine with aspartic acid at position 279 (N279D), serine with asparagine at position 280 (S280N) and valine with alanine at position 281 (V281A). However, these mutations were reversion into residues that are
commonly found in HXB2, consensus C and ancestral subtype C viruses. These findings suggested that there was immune pressure to escape bNAb specificities and this participant’s neutralization capacity may be due to the additional effects of several targeted epitopes. However, further epitope mapping experiments are required to confirm this interpretation.

5 Conclusion

In this study, we identified three individuals that developed neutralization breadth and confirmed that they developed breadth at approximately 1.5 years post-infection. Breadth and potency increased over time through mechanisms that remain undetermined. We showed that higher viral load may be important in the development of bNAbs. Two HIV-1 subtype C infected participants that developed breadth targeted epitopes that contained or are influenced by the glycans at positions 276 of loop D and 332 of the C3/V3 region of gp120 respectively. However, our data suggested that other amino acid residues or epitopes may have contributed to the neutralization breadth. This study highlighted that longer V1 domain earlier following infection may shield the V3 glycans from neutralizing antibodies, whereas shortening of the V1 domain exposes the V3 domain and might lead to the development of V3-specific antibodies. These findings are in line with previous studies that reported on the role of the V1 domain in shielding the V3 epitope (van den Kerkhof et al., 2016; Silver et al., 2019). In addition, this study also identified unusual mutations in the V2 domain and the CD4 binding site that may impact neutralization, however these will require experimental confirmation. Our study provides information that may be of important consideration in vaccine approaches.

Ethics approval and consent to participate

The study was approved by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal and the Institutional Review Board of Massachusetts General Hospital [REF:BE058/13]. The participants provided written informed consent prior to participating in the study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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**CRediT authorship contribution statement**

**Bongiwe Ndlovu:** Conceptualization, Methodology, Formal analysis, Visualization, Investigation, Writing - original draft. **Kamini Gounder:** Methodology, Formal analysis. **Daniel Muema:** Methodology. **Nagarajan Raju:** Methodology, Formal analysis. **Tandile Hermanus:** Methodology. **Qiniso Mthethwa:** Methodology. **Kim Robertson:** Methodology. **Bruce D. Walker:** Resources. **Ivelin S. Georgiev:** Resources, Methodology, Formal analysis. **Lynn Morris:** Resources, Supervision. **Penny Moore:** Conceptualization, Resources, Supervision, Writing - review & editing. **Thumbi Ndung’u:** Conceptualization, Resources, Supervision, Writing - review & editing.

**Declaration of competing interest**

None.

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**Appendix A Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2020.03.003.

**List of Abbreviations**
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