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Evolution of an HIV glycan–dependent broadly neutralizing antibody epitope through immune escape

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Abstract

Neutralizing antibodies are likely to play a crucial part in a preventative HIV-1 vaccine. Although efforts to elicit broadly cross-neutralizing (BCN) antibodies by vaccination have been unsuccessful[1-3], a minority of individuals naturally develop these antibodies after many years of infection[4-7]. How such antibodies arise, and the role of viral evolution in shaping these responses, is unknown. Here we show, in two HIV-1–infected individuals who developed BCN antibodies targeting the glycan at Asn332 on the gp120 envelope, that this glycan was absent on the initial infecting virus. However, this BCN epitope evolved within 6 months, through immune escape from earlier strain-specific antibodies that resulted in a shift of a glycan to position 332. Both viruses that lacked the glycan at amino acid 332 were resistant to the Asn332-dependent BCN monoclonal antibody PGT128 (ref. 8), whereas escaped variants that acquired this glycan were sensitive. Analysis of large sequence and neutralization data sets showed the 332 glycan to be significantly underrepresented in transmitted subtype C viruses compared to chronic viruses, with the absence of this glycan corresponding with resistance to PGT128. These findings highlight the

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Note: Supplementary information is available in the online version of the paper.

AUTHOR CONTRIBUTIONS

P.L.M. and E.S.G. designed the study, performed experiments, analyzed data and wrote the manuscript; E.S.G., C.K.W., J.N.B., T.H. and N.L.T. performed neutralization experiments and analyzed data; E.S.G., C.K.W., D.J.S., M.N., B.E.L. and N.R. generated single-genome sequences; D.J.S. and N.N. performed part of the sequence analyses; M.-R.A., L.P., R.I.S. and C.W. contributed the subtype C acute and chronic sequences; S.B. and M.S.S. designed and performed the neutralization experiments using the panel of transmitted/founder viruses; S.S.A.K., Q.A.K. and C.W. established the CAPRISA cohorts and contributed samples and data for these subjects; and L.M. designed the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

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dynamic interplay between early antibodies and viral escape in driving the evolution of conserved BCN antibody epitopes.

Although the role of glycans in shielding neutralizing epitopes has long been known[9-11], it has only recently become clear that many BCN responses directly target glycans, including the one at position 332 in the C3 region of the gp120 subunit of the HIV-1 envelope protein[8,12-18]. The recent isolation of monoclonal antibodies (mAbs) that target this glycan, which are the most potent yet described, has focused attention on this epitope[8]. These mAbs (PGT121–PGT123, PGT125–PGT128, PGT130, PGT131 and PGT135–PGT137) neutralize effectively across all HIV-1 subtypes, with the broadest, PGT128, neutralizing >70% of viruses tested[8]. Crystal structures of PGT127 and PGT128 have shown that these mAbs penetrate the glycan shield, recognizing high-mannose glycans at amino acids 301 and 332, in addition to a short β-strand in the C terminus of the V3 loop[19]. The conserved nature of these amino acids and the high potency of this class of mAbs suggest that this region may be an important vaccine target. Furthermore, this epitope is immunogenic, as Asn332-dependent BCN antibodies are often found in infected subjects who develop neutralization breadth[8,14-17]. However, as with other BCN antibodies, the factors that favor the emergence of Asn332-dependent BCN antibodies remain unclear. Here we hypothesize that the evolution of viral populations, which are under considerable immune and fitness selection pressures, creates BCN antibody epitopes essential for the development of neutralization breadth.

From a cohort of 79 HIV-1 subtype C-infected women studied starting at the point of acute infection, we focused on two participants who developed Asn332-dependent BCN antibodies. Subject CAP177 produced antibodies by 3 years after infection that were capable of neutralizing 88% of a large multisubtype panel of 225 heterologous viruses (M. Lacerda, P.L.M., N. N., M.S.S., E.S.G. *et al.*, unpublished data). The second individual, CAP314, neutralized 46% of 41 heterologous viruses after only 2 years of infection (Supplementary Fig. 1). Plasmas from CAP177 and CAP314 were unable to neutralize heterologous viruses that lacked the glycan at position 332 from the time when BCN responses were first detected and thereafter (Fig. 1a), confirming that their BCN activity depended on the 332 glycan, similar to the PGT128 mAb.

Single-genome amplification and gp160 sequencing from plasma viral RNA (Online Methods) was used to determine the amino acid sequence of the envelopes of circulating viral populations at multiple time points. This included the earliest available plasma samples, which were taken 2 weeks after infection for CAP177 (enabling inference of the transmitted/founder virus[20]) and 3 months after infection for CAP314. In both cases, these acute viruses lacked the predicted N-linked glycan at amino acid 332, although almost all sequences contained an intact glycosylation site at position 334 (Fig. 1b,c). By 5–6 months of infection, a glycan at position 332 evolved in both CAP177 and CAP314 through an N334T or N334S substitution, which also resulted in the destruction of the neighboring glycan at position 334. By 12–15 months, when BCN antibodies became detectable, the 332 glycan was present in all sequences; however, at around 2 years the glycan reverted to position 334, most likely as a consequence of viral escape from the Asn332-dependent BCN response (Fig. 1b,c). Therefore, for both CAP177 and CAP314, the glycan that formed the basis of the BCN epitope was not present on the infecting virus but evolved shortly thereafter, coincident with the appearance of strain-specific antibodies (Fig. 1c).

The 332 glycan lies within the C3 region, which is highly immunogenic in HIV-1 subtype C and is often targeted by early strain-specific antibodies[21]. Indeed, we have previously shown that in CAP177 early neutralizing antibodies targeted the C3 region[22]. As viral escape often involves glycan rearrangements[10], we postulated that the shift of a glycan from position 334

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to 332 in CAP177 and CAP314 may have been the result of neutralization escape from early strain-specific antibodies. To test this, we cloned the acute virus and a representative 6-month virus from CAP177 and CAP314 and inserted the 332 glycan into the acute clones by mutating the asparagine at position 334 to a serine (which also deleted the glycan at 334). We tested all three viruses from both individuals for sensitivity to autologous plasma from 6 months after infection. We detected autologous neutralizing responses against the acute viruses, whereas the 6-month clones were resistant to contemporaneous plasma, as expected (Fig. 2a,b). Introduction of the glycan at position 332 resulted in almost complete resistance to neutralization by plasma from CAP177 and reduced neutralization sensitivity to plasma from CAP314, suggesting that the 332 glycan evolved to afford escape from early strain-specific antibodies.

The absence of the glycan at position 332 on the acute viruses from CAP177 and CAP314 suggested that they might be resistant to BCN mAbs that depend on this glycan, such as PGT128. Neutralization experiments showed that CAP177 and CAP314 acute clones were resistant to PGT128, whereas the 6-month clones that contained the 332 glycan were highly sensitive, with a half-maximum inhibitory concentration (IC_{50}) <0.06 μg ml^{-1} (Table 1). We obtained similar data with a related mAb, PGT121, although the CAP177 acute clone showed moderate sensitivity (0.21 μg ml^{-1}) but was nevertheless 20-fold more resistant compared to the 6-month clone. Both N334S mutants (acute clones with a glycan introduced at position 332) were sensitive to PGT121 and PGT128, confirming the role of the 332 glycan in conferring sensitivity to these mAbs. None of the CAP177 or CAP314 clones was sensitive to the mAbs PGT135 or 2G12 (Table 1), which depend on additional glycans at other amino acids, that are uncommon among subtype C viruses[23-27]. Thus, whereas shifting a glycan from position 334 to 332 allowed the virus to escape autologous neutralizing antibodies, this created a new neutralizing antibody epitope that provided the antigenic stimulus to elicit BCN antibodies targeting the 332 glycan.

We assessed whether this pattern of selection of the 332 glycan was evident at a population level using more than 7,300 single-genome amplification—derived gp160 envelope sequences from acute and chronic HIV-1 infections (Online Methods)[20,28]. Sequences with evidence of dual infection were excluded. For each subject, the consensus sequence was generated and the frequency of the 332 glycan was determined. We assessed significance with Fisher's exact test. In subtype C, the 332 glycan was significantly less common among transmitted/founder viruses (45/68, 66%) compared to unmatched chronic viruses (52/62, 84%, $P = 0.0166$, Fig. 3a). To ensure that this was not due to adaption of HIV to neutralizing antibodies over the course of the epidemic time[29], we performed the same analysis using a smaller data set of 502 matched sequences from 20 individuals, with similar results ($P = 0.0457$, Fig. 3a). Although we observed the same trend in subtype B sequences, it was not statistically significant (Fig. 3a). Taken together, these results suggest that the pattern of evolution we describe for CAP177 and CAP314 may be relatively common and that the absence of the 332 glycan on subtype C viruses may provide an advantage during transmission or early viral outgrowth.

We analyzed the phenotypes of 101 transmitted/founder subtype C viruses using envelope clones generated as part of the Vaccine Immune Monitoring Core Standard Virus Panel Consortium. For this, transmitted/founder envelope sequences were inferred from singlegenome amplified and sequenced envelope amplicons derived from plasma from acutely HIV-infected subjects[30] and cloned into a mammalian expression vector. Envelope clones were transfected into 293T cells with the HIV backbone construct pSG3ΔEnv to produce envelope pseudotyped particles, and neutralization assays were performed in TZM-bl cells as described in the Online Methods. Phenotypic analysis supported the genotypic analysis, with a high proportion (46%) of viruses resistant to PGT128 neutralization at the

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highest concentration tested (10 μg ml^{-1}) (Fig. 3b and Supplementary Fig. 2). Resistance strongly correlated with the absence of the 332 glycan ($P < 0.0001$) (Fig. 3c), although some viruses that contained the glycan were also resistant, consistent with the fact that additional residues are needed to form this epitope[8]. Of 31 viruses in which the glycan at position 332 was absent, only three showed neutralization sensitivity. Of these, two contained the glycan at position 295, which is very rare in subtype C viruses[26] but structurally proximal to the 332 glycan and shown by mutagenesis to affect the PGT128 epitope[19]. Although this virus panel was tested only against PGT128, resistance to this mAb generally extends to other Asn332-dependent PGT mAbs[8]. These data suggest that Asn332-dependent antibodies present either through passive immunotherapy or vaccination might be only partially effective in preventing subtype C infections and that combinations of antibodies targeting different epitopes may need to be tailored to match circulating viral variants[24,26,31].

In addition to the Asn332-dependent epitope, a second BCN antibody epitope that includes the glycans at amino acid positions 156 and 160 in the V2 region has been defined by the mAbs PG9, PG16 (ref. 18) and PGT145 (ref. 8). To assess whether epitope evolution was also associated with the appearance of Asn160-dependent BCN antibodies, we studied two individuals, CAP8 and CAP256, who developed this specificity[15] (Supplementary Fig. 3a). In both cases, the glycan at amino acid 160 was absent from envelope sequences at transmission but appeared at 6 and 3 months in CAP8 and CAP256, respectively, suggesting a pattern of evolution similar to that observed with 332 glycan (Supplementary Fig. 3b). Furthermore, we found acute viruses to be resistant to BCN mAbs PG9, PG16 and PGT145, whereas later clones that contained the 160 glycan became sensitive to PG9 and PGT145, although not to PG16 (Supplementary Fig. 4). However, although insertion of the 160 glycan by site-directed mutagenesis into the resistant acute clones rendered them sensitive to PG9 and PGT145, unlike the case with the CAP177 and CAP314 mutants containing the 332 glycan, this single change did not mediate neutralization escape. It was also not possible to show selection of the 160 glycan using large sequence data sets, as it is highly conserved in both acute and chronic sequences (Supplementary Fig. 5).

Despite the fact that BCN epitopes are highly conserved, they only occasionally elicit BCN responses in humans. Those targeting epitopes centered around the 332 or 160 glycans develop more frequently in infected people compared to BCN antibodies directed at the CD4 binding site or membrane-proximal region[12-14]. The data presented here raise the hypothesis that, at least in the case of Asn322-directed antibodies, viral evolution may facilitate their elicitation. However, the absence of these glycans on the acute virus is not a prerequisite for the development of breadth, nor does the evolution of a BCN epitope guarantee that these antibodies will arise. Indeed, within this cohort, examples of both scenarios exist (Supplementary Fig. 6a,b). Furthermore, in a study of a SHIV-infected macaque[32] where the 332 glycan was present at transmission, potent and broad Asn332-dependent BCN antibodies developed within 9 months. In HIV-1 subtype C viruses, where the absence of the 332 glycan is favored at transmission, subsequent immune pressure exerted by strain-specific neutralizing antibodies results in the evolution of the 332 glycan. This process provides a mechanism for the evolution of BCN epitopes, with neutralization escape driving viral convergence toward glycan motifs that are highly conserved and serve as targets for BCN antibodies.

ONLINE METHODS

Participants

CAP177, CAP8 and CAP256 were part of the CAPRISA 002 Acute Infection study, a cohort of 245 high-risk, HIV-negative women that was established in 2004 in Durban, South Africa, for follow-up and subsequent identification of HIV seroconversion[33]. These three

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HIV-infected subjects were among the seven women in this cohort who developed neutralization breadth[15]. The fourth individual, CAP314, was enrolled in the CAPRISA 004 trial, a two-arm, double-blind, randomized, placebo-controlled trial, conducted from May 2007 to March 2010, to assess the effectiveness and safety of tenofovir gel for the prevention of HIV infection in women[34]. Monitoring BCN antibodies in this cohort of 39 women showed that she was one of three who developed breadth by 2 years of infection, two of whom targeted an Asn332-dependent epitope. The CAPRISA 002 Acute Infection study was reviewed and approved by the research ethics committees of the University of KwaZulu-Natal (E013/04), the University of Cape Town (025/2004) and the University of the Witwatersrand (MM040202). The CAPRISA 004 trial was approved by the University of KwaZulu-Natal's Biomedical Research Ethics Committee (E111/06), Family Health International's Protection of Human Subjects Committee (9946) and the South African Medicines Control Council (20060835). All participants provided written informed consent.

Single-genome amplification and sequencing

HIV-1 RNA was isolated from plasma using the Qiagen QIAamp Viral RNA kit and reverse transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen, CA). The envelope genes were amplified from single-genome templates[30], and amplicons were directly sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) and resolved on an ABI 3100 automated genetic analyzer. The full-length *env* sequences were assembled and edited using Sequencher version 4.0 software (Genecodes, Ann Arbor, MI). The number of potential N-linked glycosylation sites was determined using N-glycosite (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html/). Multiple sequence alignments were performed using Clustal X (version 1.83) and edited with BioEdit (version 5.0.9) Sequence alignments were visualized using Highlighter for Amino Acid Sequences version 1.1.0 (beta). Selected amplicons were cloned into the expression vector pcDNA 3.1 (directional) (Invitrogen) by reamplification of SGA first-round products using Phusion enzyme (Finn Enzymes) with the EnvM primer[35] and the directional primer EnvAdir[21]. Cloned *env* genes were sequenced to confirm that they exactly matched the sequenced amplicon.

Site-directed mutagenesis

Sensitive heterologous viruses Q23, Du156, TRO.11, CAP200 and ConC were mutated at key residues (332–334 or 160–162) by site-directed mutagenesis using the Stratagene QuickChange II kit (Stratagene) as described by the manufacturer. Mutations were confirmed by sequencing. Autologous clones derived from CAP177 and CAP314 were mutated to shift the glycan from position 334 to 332, and mutants were verified by sequencing. Similarly autologous clones from CAP8 and CAP256 were mutated to introduce the glycan at position 160, and mutants were verified by sequencing.

Neutralization assays

The JC53bl-13 (TZM-bl) cell line was obtained from the NIH AIDS Research and Reference Reagent Program. 293T cells were obtained from G. Shaw. Both cell lines were cultured in DMEM (Gibco BRL Life Technologies) containing 10% heat-inactivated FBS and 50 μg/ml gentamicin (Sigma). Cell monolayers were disrupted at confluency by treatment with 0.25% trypsin in 1mM EDTA. Env-pseudotyped viruses were obtained by co-transfecting the Env plasmid with pSG3ΔEnv[10] using FuGENE transfection reagent (Roche) as previously described[36]. Neutralization was measured as described[36] by a reduction in luciferase gene expression after single-round infection of JC53bl-13 cells with Env-pseudotyped viruses. Titers were calculated as the reciprocal plasma dilution (ID_{50}) causing 50% reduction of relative light units (RLU).

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Supplementary Material

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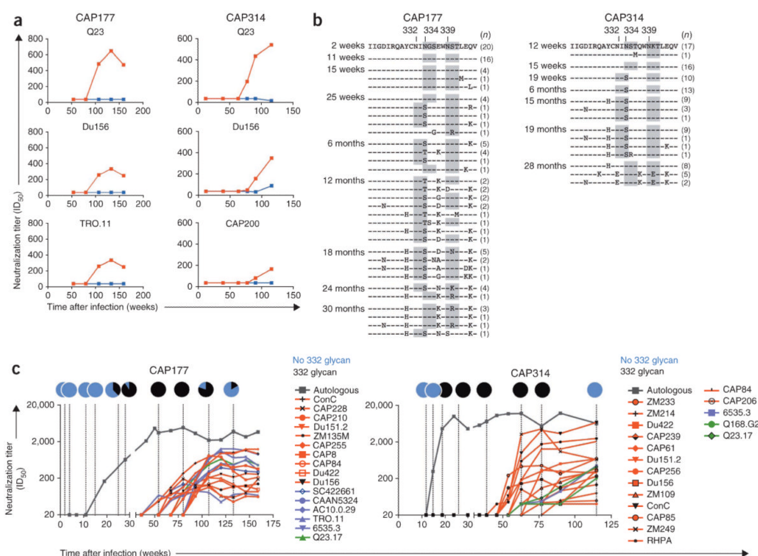

Figure 1.

Characterizing the antibody specificities and viral populations in CAP177 and CAP314. (**a**) Serial plasma neutralization of three heterologous viruses (Q23, Du156 and TRO.11 for CAP177 and Q23, Du156 and CAP200 for CAP314) compared to mutant viruses where the 332 glycan was deleted by mutagenesis. Wild-type viruses are shown in orange and mutants in blue. (**b**) Sequence alignment showing part of the C3 region from 2 weeks (CAP177) or 3 months (CAP314) up to 28–30 months after infection. The number of sequences at each time point is indicated. Gray shading indicates the presence of a predicted N-linked glycan, and dashes indicate sequence identity compared to the acute sequence. HXB2 numbering is used throughout. (**c**) The frequency of the 332 glycan is shown over time (as pie charts) relative to neutralization of the acute autologous virus (corresponding to the 2-week and 3-month sequences shown above, gray line) and sensitive heterologous viruses (of subtype C in orange, subtype B in blue and subtype A in green). Plasma samples were available only up to 2 years after infection for CAP314, who received antiretroviral therapy shortly thereafter.

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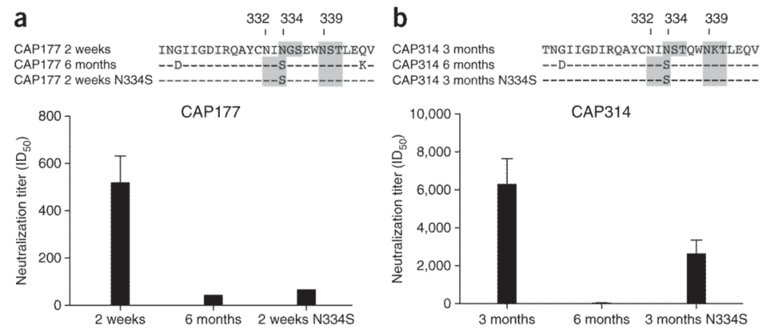

Figure 2.

The glycan at position 332 mediates neutralization escape in CAP177 and CAP314. (**a**) Partial sequence alignment showing the CAP177 acute virus (CAP177 2 weeks), a representative 6-month clone (CAP177 6 months) and the acute virus mutated to contain a glycan at position 332, which deletes the glycan at position 334 (CAP177 2 weeks N334S). All three clones were tested for neutralization sensitivity to autologous serum from 6 months after infection. (**b**) Partial sequence alignment showing the CAP314 acute virus (CAP314 3 months), a representative 6-month clone (CAP314 6 months) and the mutated virus (CAP314 3 months N334S) and their sensitivity to autologous serum from 6 months after infection. Residual neutralization of the CAP314 3 months N334S mutant reflects the presence of neutralizing antibody specificities targeting other epitopes, as described previously[22]. Experiments were performed three times, and error bars represent the s.e.m.

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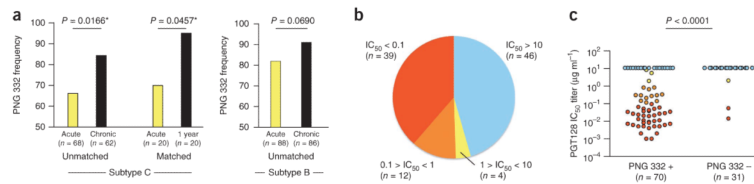

Figure 3.

The glycan at residue 332 is underrepresented in subtype C transmitted/founder viruses, which are also frequently resistant to the PGT128 mAb. (**a**) Comparison of the frequency of the 332 glycan among 1,371 envelope sequences from 68 subjects with HIV-1 subtype C acute or early infection[20] and 1,111 sequences from 62 subjects with chronic HIV-1 subtype C infections (L.P., S. Joseph, J. Anderson, M.-R.A., J. Salazar-Gonzalez *et al.*, unpublished data). We also analyzed a smaller data set of matched sequences (502 sequences from 20 subjects from the CAPRISA cohort), and compared acute sequences to those from the same individual at 1 year after infection. For subtype B, a published data set of 2,715 acute sequences from 88 subjects and 2,122 sequences from 86 chronic infections was used[28]. (**b**) Neutralization sensitivity of 101 transmitted/founder viruses to neutralization by the PGT128 mAb. (**c**) Neutralization sensitivity of subtype C transmitted/founder viruses correlated strongly with the presence or absence of the 332 glycan. Colors reflect neutralization IC_{50} titers >10 μg ml^{-1} (blue), 1–10 μg ml^{-1} (yellow), 0.1–1μg ml^{-1} (orange) and <0.1 μg ml^{-1} (red).

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Table 1

Neutralization of CAP177 and CAP314 pseudoviruses by BCN monoclonal antibodies

mAb (IC_{50} titers)	PNG 332	PGT121	PGT128	PGT135	2G12
CAP177 2 weeks	–	0.21	>10	>10	>10
CAP177 6 months	+	0.01	0.06	>10	>10
CAP177 2 weeks N334S	+	0.04	0.71	>10	>10
CAP314 3 months	–	>10	>10	>10	>10
CAP314 6 months	+	0.03	0.03	>10	>10
CAP314 3 months N334S	+	0.03	0.07	>10	>10
TRO.11 (control)	+	0.03	0.06	0.16	0.49
Donor subtype		A	CRF02_AG	C	B
Breadth[a]		70%[8]	72%[8]	33%[8]	41%[24]

[a]Percentage of viruses neutralized with IC_{50} <50 μg ml^{-1}. Sensitivity of CAP177 and CAP314 clones to neutralization by Asn332-dependent BCN monoclonal antibodies. The acute, 6-month and N334S mutant viruses from both CAP177 and CAP314 were tested against mAbs PGT121, PGT128, PGT135 and 2G12 in a TZM-bl neutralization assay. Colors reflect neutralization IC50 titers >10 μg ml^{-1} (blue), 0.1–10 μg ml^{-1} (orange) and <0.1 μg ml^{-1} (red). TRO.11, a subtype B virus containing the glycan at residue 332, was included as a positive control. For the mAbs, the subtype of the donor from which they were isolated together with published data on their neutralization breadth is also included.

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