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Viral variants that initiate and drive maturation of V1V2-directed HIV-1 broadly neutralizing antibodies

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Abstract

The elicitation of broadly neutralizing antibodies (bNAbs) is likely to be essential for a preventative HIV-1 vaccine, but this has not yet been achieved by immunization. In contrast some HIV-1-infected individuals naturally mount bNAb responses during chronic infection, suggesting that years of maturation are required for breadth¹⁻⁶. Recent studies have shown that viral diversification precedes the emergence of bNAbs but the significance of this observation is unknown^{7,8}. Here, we delineate the key viral events that drove neutralization breadth within the

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AUTHOR CONTRIBUTIONS

J.N.B., L.M. and P.L.M. designed the study, analysed data and wrote the manuscript. C.A. and C.W. designed and performed viral next-generation sequencing and analysis and edited the manuscript. N.A.D-R led the isolation of CAP256 mAbs. J.N.B. and O.K. performed neutralization experiments. C.A.S. and L.S. generated and analysed antibody NGS data. J.N.B. and T.K. expressed and purified monoclonal antibodies. J.N.B. and D.K. generated single-genome sequences and viral envelope clones. G.B. developed computational tools to analyse NGS data, and assisted in the NGS analysis. J.G. assessed CAP256-VRC26 UCA neutralization of heterologous viruses. N.J.G. and the CAPRISA Study Team managed the CAPRISA cohort and contributed samples and data for CAP256. S.S.A.K., C.W. and L.M. established and led the CAPRISA cohort. C.W., N.A.D-R, L.S, P.D.K., and J.R.M contributed to data analysis and edited the manuscript.

CONFLICTING FINANCIAL INTERESTS

The authors declare no competing financial interests.

CAP256-VRC26 family of 33 monoclonal antibodies (mAbs) isolated from a superinfected individual. First, we identified minority viral variants that were distinct from both transmitted/founder (T/F) viruses and efficiently engaged the bNAb precursor, termed bNAb-initiating envelopes. Second, deep sequencing revealed a pool of diverse epitope variants (immunotypes) that were preferentially neutralized by broader members of the antibody lineage. In contrast, a “dead-end” antibody sublineage unable to neutralize these immunotypes showed limited evolution and failed to develop breadth. Thus, early viral escape at key antibody-virus contact sites selects for sublineages that can tolerate these changes, providing a new mechanism for the generation of neutralization breadth within a developing antibody lineage.

The identification of bNAbs against genetically diverse viruses such as HIV-1⁹, together with in-depth longitudinal evolutionary studies are providing key insights for vaccine design^{7,8,10-12}. bNAbs to the variable regions 1 and 2 (V1V2) of the HIV-1 envelope (Env) are among the most prevalent and potent cross-reactive antibodies, but the virological events that allow for their elicitation and maturation remain unclear. We have previously described the CAP256-VRC26 mAb lineage, isolated from an HIV-1 subtype C superinfected individual, which targets the V1V2 C-strand at the apex of the viral envelope glycoprotein (Fig. 1a)⁷. This lineage developed from a bNAb precursor or unmutated common ancestor (UCA) with a 35-amino acid heavy chain complementarity determining region three (CDRH3), and acquired breadth through moderate levels of somatic hypermutation (SHM)⁷. To better define the complex viral populations responsible for eliciting these antibodies and driving their maturation, we performed next-generation sequencing (NGS) at 28 different time-points over four years (with an average of 85,000 reads and 788 consensus sequences per time-point). From 6–13 weeks, all sequences were closely related to the primary infecting virus (PI) (Fig. 1b and Supplementary Fig. 1) but at 15 weeks, when superinfection (SU) occurred, there was a dramatic shift in viral populations, with SU-like viruses accounting for 99.8 % of 3,228 consensus sequences (Supplementary Fig. 2). From 17 weeks, V1V2 sequences from the PI and SU viruses persisted, rapidly forming a PI-SU recombinant population, although PI-like viruses dominated briefly at 23 weeks, perhaps a result of immune escape from earlier non-V1V2 directed neutralizing antibodies that preferentially neutralized SU-like viruses¹³. Notably, diversification in all three viral populations (PI, SU and recombinants) increased after the CAP256-VRC26 lineage was first detected in blood by NGS at 34 weeks (Fig. 1b, gray shading and Supplementary Fig. 3).

As residues 169 and 166 in the V1V2 C-strand form a crucial part of the CAP256-VRC26 bNAb epitope and would likely be important in virus-mAb co-evolution, we focused on defining escape mutations at these positions. Residue 169 differed between the PI (169Q) and SU (169K), with the majority of viral variants circulating after 23 weeks possessing a PI-derived 169Q (Fig. 1c and Supplementary Fig. 2). From 27 weeks onwards, the virus sampled 11 different amino acids at position 169, with variation most pronounced at 42 weeks (Fig. 1c **inset**), shortly before the development of breadth. The major escape pathway from 42–119 weeks was a 169I mutation, and thereafter 169E dominated. Toggling was also observed at position 166 (Fig. 1d), though this occurred later, with the wildtype 166R immunotype (present in both the PI and SU) found in more than 98% of sequences until 53 weeks. At 59 weeks, the virus sampled four amino acids (166R, T, K or S) and thereafter

166K, followed by 166S, became prevalent. By 159 weeks, all viral sequences contained the mutually exclusive K169E or R166S mutations. As both K169E and R166S abrogate neutralization by the CAP256-VRC26 mAbs^{7,13} it is unclear why it took two years for these escape pathways to dominate despite their low-level presence early in the development of the CAP256-VRC26 family (Supplementary Fig. 2).

The CAP256-VRC26 UCA was unable to neutralize any of 196 HIV-1 isolates (Supplementary Fig. 4) suggesting that Env variants able to engage this UCA may be rare. Since CAP256-VRC26 NGS transcripts were first detected in blood 19 weeks after superinfection (Supplementary Fig. 3), we analyzed contemporaneous (34 week) viral sequences to identify a bNAb-initiating Env. Neutralization of the SU, but not the PI virus, by the CAP256-VRC26 UCA indicated that an SU-like variant elicited this lineage⁷. Although 8% (75/937) of V1V2 next-generation sequences at the 34 week time-point were SU-like, none were identical to the SU (Fig. 2a and Supplementary Fig. 5). Single genome amplification was therefore used to isolate six functional 34-week Env clones, which differed from the SU by two to eight amino acids in V1V2 and by up to 26 changes across the entire Env (Fig. 2b). There was evidence of early immune pressure, with three of six clones containing K169I or E mutations, and four of six clones deleting or shifting the glycan at residue 139 (Fig. 2c). As expected, the clones with 169I or E mutations were resistant to neutralization by the UCA, however the three clones containing a wild-type 169K were 5–17 fold better neutralized than the SU virus (Fig. 2d). Thus, the 34-week viral variants that evolved from the CAP256 SU T/F virus (but differed from the T/F by at least 12 amino acids) provided a stronger stimulus for the CAP256-VRC26 lineage.

We next sought to define how this lineage matured to acquire neutralization breadth in the context of CAP256 viral diversification. A heavy chain phylogenetic tree of 33 isolated CAP256-VRC26 mAbs (12 previously described⁷ and 21 recently isolated mAbs, N.D-R. and J.R.M. unpublished data) and CAP256-VRC26 NGS transcripts from 34 to 206 weeks showed an early bifurcation⁷ (Fig. 3a). A small sublineage of 34–59 week sequences including the CAP256-VRC26.01 and .24 mAbs exhibited restricted evolution. A second larger sublineage, containing transcripts from all time-points as well as most of the mAbs (Fig. 3a), displayed extensive evolution suggesting adaption in response to emerging viral escape mutations, such as K169T/I/Q/R and R166K (Fig 1c, d). In order to test this hypothesis, we introduced these immunotypes into the SU virus and tested them against 32 mAbs (CAP256-VRC26.20 does not neutralize SU and was excluded). MAbs CAP256-VRC26.01 and .24, from the smaller sublineage with limited breadth, both neutralized the SU at 0.04 µg/ml (Fig. 3b). However SU viruses containing the 166 or 169 immunotypes were either neutralization resistant or showed a >670-fold reduction in neutralization sensitivity (Fig. 3b). Thus, the inability of mAbs in this “dead-end” sublineage to tolerate viral escape mutations appeared to be responsible for limiting their evolution and capacity to become broadly neutralizing.

Among the 30 CAP256-VRC26 mAbs from the evolving sublineage that showed a range of neutralization breadth (2–63%), the 169T, I or Q mutations abolished or reduced the activity of mAbs with limited breadth (Fig. 3c). In contrast, the neutralizing activity of broader family members was largely unaffected. This observation was more pronounced for the

R166K mutant, which was resistant to 16/30 mAbs (Fig. 3c), consistent with the selection of this immunotype during later stages of bNAb development (Fig. 1d). However, six of the nine broadest mAbs were still able to neutralize this mutant at titers $<1 \mu\text{g/ml}$. With the exception of the K169R mutation, breadth was correlated with the ability to neutralize the 169 or 166 mutants (Fig. 3c and Supplementary Fig. 6). Overall mAbs with limited neutralization breadth were most severely affected by variation at positions 169 and 166, while the broadest mAbs were able to tolerate these early viral escape mutations, providing a mechanism for the evolution of neutralization breadth within this lineage.

Since high levels of SHM are often required for HIV-1 broad neutralization^{14,15}, we investigated the relationship between SHM and neutralization breadth in the CAP256-VRC26 family (Fig. 4a). As expected, the nine mAbs with $>40\%$ breadth were 17–22% mutated from the UCA, however three “off-track” mAbs with a similar level of SHM (17–21%) had only 2–6% breadth (Fig. 4a). Therefore, as observed in several families of anti-HIV antibodies¹⁶⁻¹⁹ and anti-influenza antibodies²⁰, high levels of SHM in the CAP256-VRC26 lineage were not sufficient to confer breadth. To determine if different viral variants drove the evolution of broad versus off-track mAbs, we tested both groups of mAbs against longitudinal CAP256 viruses (Fig. 4b). Hierarchical clustering analysis of mAbs and viruses based on their neutralization profiles showed a clear phenotypic separation between broad and off-track mAbs with the latter neutralizing substantially fewer autologous viruses, but we could not attribute this to any genetic features of the mAbs. Both groups neutralized viruses with an SU-like C-strand, including the bNAb-initiating 34-week clones. All viruses neutralized by off-track antibodies were also sensitive to at least one broad mAb, suggesting that viral variants associated with the development of neutralization breadth also selected off-track mAbs. Indeed, clustering of broad and off-track mAbs within a single phylogenetic branch highlights the stochastic nature of mAb maturation towards breadth (Fig. 4c).

In conclusion, we identified 34-week viral variants that effectively engaged the UCA of the CAP256-VRC26 V1V2 lineage, which we have termed bNAb-initiating Envs. While low frequency viral escape mutations at 27–30 weeks may suggest that CAP256-VRC26 antibodies were present earlier, the 34-week Envs nonetheless represent optimal priming immunogens for this lineage. Identification of rare bNAb-initiating Envs is likely to be of particular significance for V1V2 bNAb precursors which are derived from a pool of rare B cells with long CDRH3s^{7,17,21-25}. Secondly, we have defined a new mechanism for how accumulating viral variants contribute to bNAb development. Thus, viral escape creates multiple immunotypes, and in parallel SHM generates antibodies with differential ability to engage these epitope variants. Those antibody sublineages able to tolerate variability at key epitope contacts will develop breadth (Fig. 4d). We note however that viral diversity may also limit the evolution of less tolerant mAbs (dead-end sublineages), or may divert maturation of mAbs towards strain-specificity (off-track mAbs) (Fig. 4d). This process, which provides a framework for vaccine design (Supplementary Fig. 7), is distinct from the multi-lineage cooperative pathway described for a CD4 binding site bNAb lineage¹⁰, highlighting the complex and varied pathways to breadth. Future immunization strategies should therefore aim to include bNAb-initiating Envs to prime bNAb precursor B-cells, as well as rationally verified Env variants as sequential boosts to mature the response towards neutralization breadth.

ONLINE METHODS

Study participant

CAP256 was 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 seroconversion²⁶. This HIV-infected individual was among the seven women in this cohort who developed neutralization breadth¹. 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). CAP256 provided written informed consent. Randomization and blinding were not performed in this study.

V1V2 next-generation sequence library preparation

RNA extraction, cDNA synthesis and subsequent amplification were carried out as described previously²⁷, with the following modifications: A minimum of 5,000 HIV-1 RNA copies were isolated from longitudinal plasma, spanning four years of infection, using the QIAamp Viral RNA kit (Qiagen, Germany). The cDNA synthesis primer was designed to bind to the C3 region of the envelope (HXB2 position 7,342–7,318) and included a randomly assigned 9-mer tag (Primer ID method) to uniquely label each cDNA molecule, followed by a universal primer binding site to allow out-nested PCR amplification of cDNA templates (primers listed in Supplementary Table 1). First round amplification primers were designed to amplify the V1 to V3 region of the envelope (HXB2 positions 6,556 to 7,342) and contained a template specific binding region, followed by a variable length spacer of zero to three randomly assigned bases to increase sample complexity. In addition, PCR primers contained 5' overhangs, introducing binding sites for the Nextera XT indexing primers (Illumina, CA) (Supplementary Table 1). The nested PCR reactions were carried out using the Nextera XT indexing kit. Following indexing, samples were purified using the MinElute PCR Purification Kit (Qiagen, Germany), quantified using the Quant-iT PicoGreen dsDNA assay (Life Technologies, NY) and pooled in equimolar concentrations. The pooled amplicons were gel extracted using the QIAquick gel purification kit (Qiagen, Germany) to ensure primer removal and the final library submitted for sequencing on an Illumina MiSeq (San Diego, CA), using 2×300 paired-end chemistry.

V1V2 next-generation sequence analysis

Raw reads were processed using a local instance of Galaxy²⁸⁻³⁰, housed within the University of Cape Town High Performance Computing core. Read quality was assessed using fastqc (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Short reads (<150 bp) and low quality data were filtered out using the Filter FASTQ (version 1.0.0) tool³¹ with a minimum quality of Q35 for 3' base trimming. Forward and reverse reads were joined using FASTQ joiner (version 2.0.0)³¹. A custom python script was used to bin all reads containing an identical Primer ID tag, align the reads within each bin using MAFFT³² and produce a consensus sequence based on a majority rule. Sequences with just one Primer ID representative, as well as consensus sequences derived from less than three reads and those containing degenerate bases, were excluded and the remaining sequences from each time point aligned with MAFFT, Muscle³³ or RAMICS³⁴. The calculation of amino acid

frequencies and hamming distances (for each sequence relative to the consensus sequence from the first time point –inferred PI virus), were performed using custom python scripts. Amino acid frequency distributions were plotted using Prism 6 (GraphPad), while hamming distances were plotted using the python library Matplotlib³⁵. All custom scripts used in these analyses, including the Primer ID binning script, are available upon request.

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 templates³⁶, and amplicons were directly sequenced using the ABI PRISM BigDye 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 4.5 software (Genecodes, Ann Arbor, MI). Multiple sequence alignments were performed using Clustal X (version 1.83) and edited with BioEdit (version 7.0.9.0). 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) (Life Technologies) by reamplification of SGA first-round products using Fusion enzyme (Stratagene) with the EnvM primer³⁷ and the directional primer EnvAStop³⁸. Cloned *env* genes were sequenced to confirm that they exactly matched the sequenced amplicon, and assayed for function by transfection in 293T cells, as described below.

Site-directed mutagenesis

CAP256 15-wk SU was mutated at positions 169 and 166 by site-directed mutagenesis using the Stratagene QuikChange II kit (Stratagene) as described by the manufacturer. Mutations were confirmed by sequencing.

Antibody next-generation sequence analysis

NGS reads from each time point were processed with an antibodyomics described previously⁷. Reads with successfully assigned V and J genes, an in-frame junction and no stop codons were clustered at 97.25 % nucleotide sequence identity with CD-HIT to account for possible sequencing error and singletons were discarded. For those remaining reads assigned to VH3-30, divergence from VH and identity to the previously determined UCA⁷ were calculated with Clustalw2. Density plots for these sequences were generated using the kde2d function in R. A maximum likelihood tree was built using FastML as described⁷. The tree was collapsed for display by clustering CDRH3 sequences as described⁷ using a 90% identity threshold and a minimum of three sequences to define a major branch.

Antibody expression

Equal quantities of antibody heavy and light chain plasmids were co-transfected into FreeStyle™ 293F cells (Life Technologies) using the PEIMAX transfection reagent (polysciences). Monoclonal antibodies were purified from cell-free supernatants after six days using protein A affinity chromatography.

Neutralization assays

The JC53bl-13 (TZM-bl) and 293T cell lines were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr John C. Kappes, Dr Xiaoyun Wu and Tranzyme Inc. and Dr Andrew Rice respectively. Both cell lines, confirmed to be free of *Mycoplasma*, 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 (Gibco BRL Life Technologies). Env-pseudotyped viruses were obtained by co-transfecting the Env plasmid with pSG3 Env³⁹ using FuGENE transfection reagent (Roche) as previously described¹. Neutralization was measured as described by a reduction in luciferase gene expression after single-round infection of JC53bl-13 cells with Env-pseudotyped viruses¹. Titers were calculated as the reciprocal plasma dilution (ID₅₀) causing 50% reduction of relative light units (RLU). Viral and antibody hierarchical clustering based on neutralization profiles was performed using the Heatmap Hierarchical Clustering tool on the LANL HIV sequence database <http://www.hiv.lanl.gov/content/sequence/HEATMAP/heatmap.html>.

Images

Electron microscopy and protein structure representations were created with Chimera 1.5.3rc and PyMOL 1.3⁴⁰. Neutralization images were created in GraphPad Prism6, sequence alignments were made with BioEdit⁴¹ and phylogenetic trees were edited in FigTree 1.4.2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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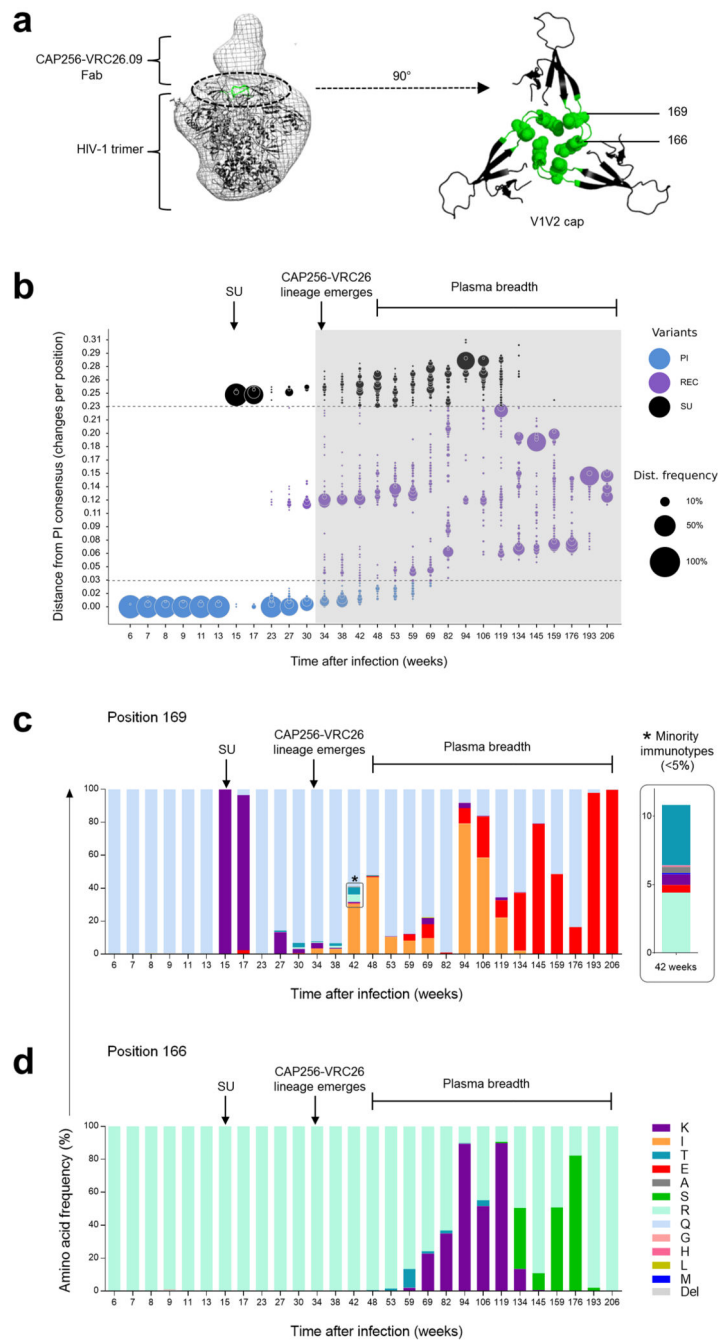


Figure 1.

Viral diversification over time in CAP256 HIV-1 Env V1V2. **(a)** BG505 SOSIP.664 trimer structure (PDB:4TVP) fitted into a 3D reconstruction of the CAP256-VRC26.09 Fab-trimer complex (EMD-5856, left panel). BG505 V1V2 cap with residues 166 and 169 shown as spheres (right panel) and the C-strand highlighted in green. **(b)** Hamming distance from the PI sequence (y-axis) versus weeks after infection (x-axis) for all next-generation V1V2 consensus sequences. Criteria of <0.03 and >0.23 were used to distinguish PI-like (blue) and SU-like (black) viruses respectively, while those sequences with an intermediate distance

(0.05–0.21) were classified as PI-SU recombinant viruses (purple, REC). Hamming distances were normalized for sequence length, and the relative frequency of sequences with a given distance are indicated by the size of the circle (normalized for depth of sequencing, but not for viral load, see Supplementary Figure 1). CAP256-VRC26 next generation transcript detection in the blood is shown by gray shading. (c and d) The frequency of amino acids (y-axis) at positions 169 (c) and 166 (d) are shown as stacked bars, with each bar representing a single time-point from 6 to 206 weeks after infection (x-axis). Amino acids are colored as indicated in the key, with Del representing a deletion. The inset (*) in the 169 graph expands the 42 week time-point (when nine immunotypes are present), to highlight the seven minority immunotypes present at frequencies of <5%. The time of superinfection (SU), the emergence of CAP256-VRC26 NGS transcripts and onset of plasma breadth are shown.

UCA mAb concentration is shown on the x-axis. Bars indicate standard error of the mean (triplicate).

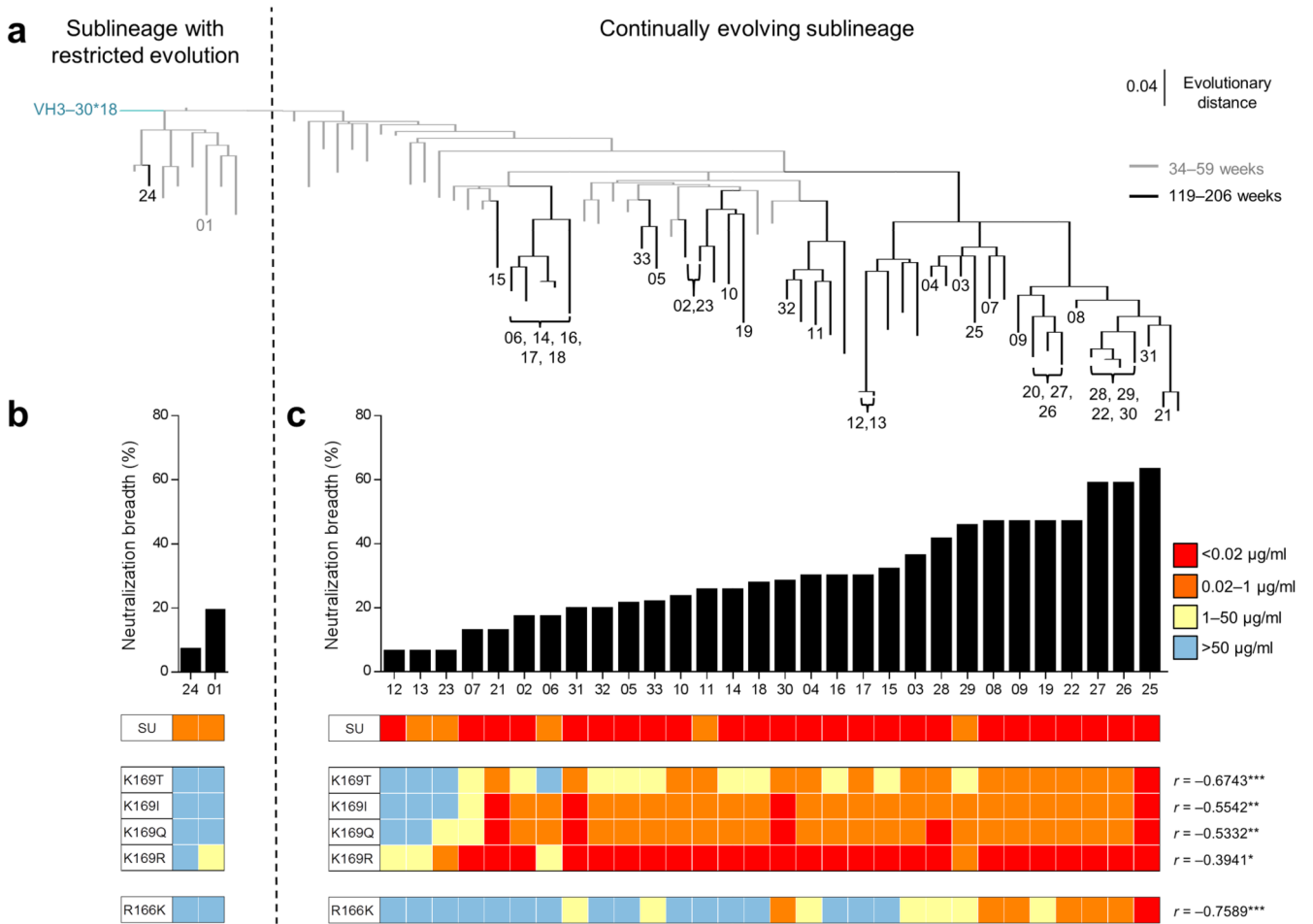


Figure 3.

Relationship between bNAb phylogeny, neutralization breadth and tolerance of multiple immunotypes at positions 169 and 166. **(a)** A condensed heavy chain phylogenetic tree, rooted on the VH3-30*18 germline gene and displaying the positions of the 33 CAP256-VRC26 mAbs. Antibody transcripts and each mAb is colored according to whether it was isolated from 34–59 weeks (gray) or 119–206 weeks (black). The tree bifurcates into two sublineages (separated by a dashed line); one showing restricted evolution (left) contains only antibody transcripts from early time-points, while a continually evolving sublineage (right) contains transcripts from all time-points. Scale shows the rate of nucleotide change between nodes. **(b)** Dead-end mAbs in the sublineage with restricted evolution are ranked according to percentage neutralization breadth in the histogram. Neutralization of the SU virus, 169 and 166 mutant viruses by CAP256-VRC26.01 and .24 are shown as a heatmap, with shading showing neutralization potency, as indicated in the key. **(c)** Thirty mAbs in the continually evolving sublineage are ranked according to neutralization breadth in the histogram. Neutralization of the SU virus, 169 and 166 mutant viruses by CAP256-VRC26 mAbs are shown as a heatmap, with shading showing neutralization potency, as indicated in the key. Correlations (r values) between neutralization breadth and the IC_{50} titres of each mutant virus are indicated on the right of the heatmap with P values shown as stars ($*P <$

0.05; ** $P < 0.005$; *** $P < 0.0001$ calculated using a two-tailed non-parametric Spearman correlation).

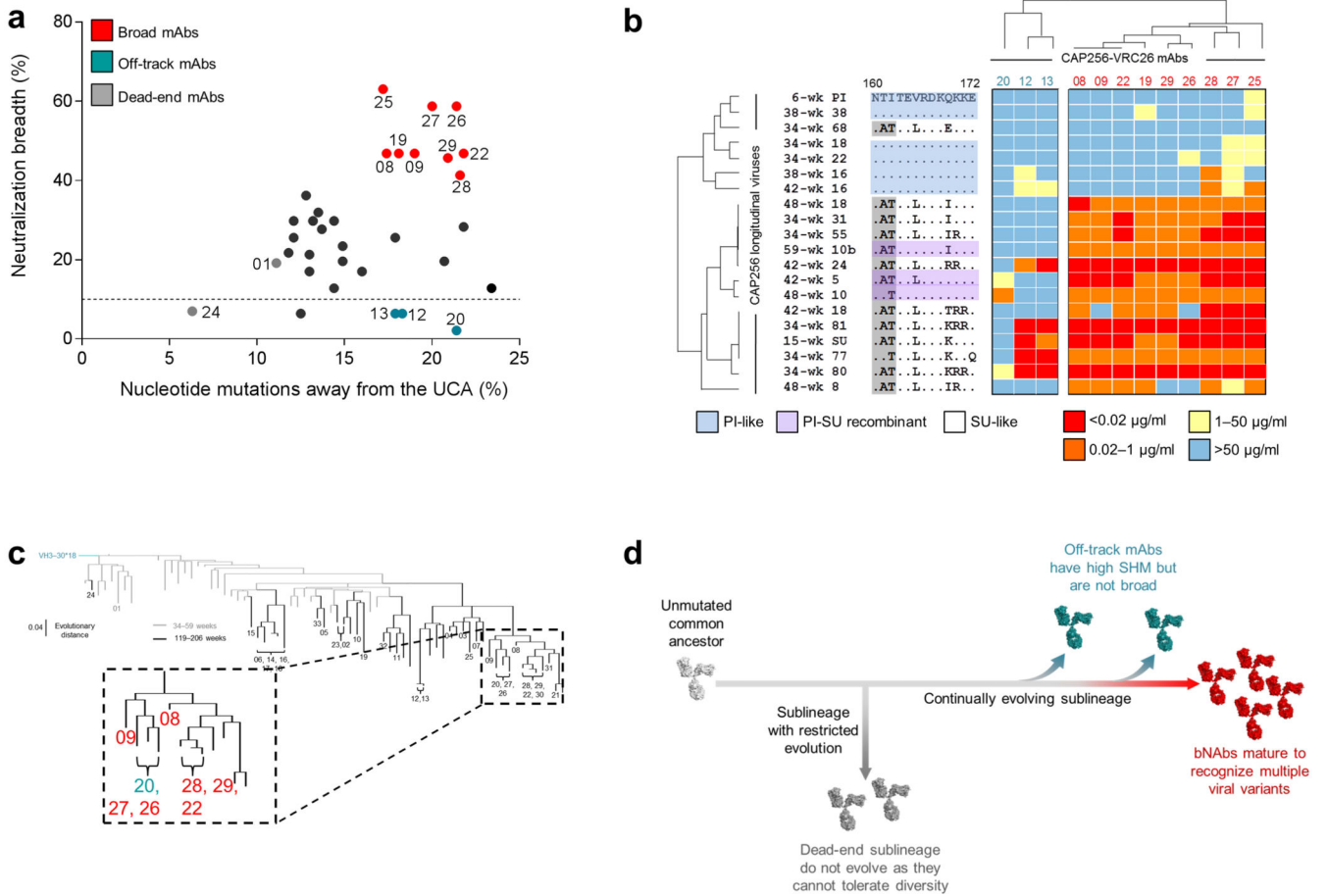


Figure 4.

Viral variants that shaped the maturation of broad and off-track antibodies. **(a)** Relationship between neutralization breadth of CAP256-VRC26 mAbs (% , y-axis) and mutations away from the UCA (% , x-axis). Broad mAbs with >40% neutralization breadth, off-track mAbs with limited neutralization breadth (<6%) and dead-end mAbs are shown in red, turquoise and gray respectively. **(b)** CAP256-VRC26 mAbs and longitudinal CAP256 viruses isolated between 6–59 weeks are hierarchically clustered by their neutralization profiles. Neutralization sensitivity is shown as a heatmap with potency indicated in the key. The sequence alignment shows viral C-strand amino acid residues between positions 160–172 (HXB2 numbering), shaded to designate sequences that are PI-derived (blue shading), PI-SU recombinants (purple shading) or SU-derived (no shading). Off-track (turquoise) and broad (red) mAbs are clustered separately, while longitudinal viral variants are clustered by neutralization sensitivity, largely segregated on the basis of an SU-derived V1V2 C-strand. **(c)** A condensed heavy chain phylogenetic tree (Figure 3) with a single clade expanded to illustrate the phylogenetic relatedness of an off-track mAb (turquoise) to seven broad mAbs (red). **(d)** CAP256 viral evolution results in the engagement of the CAP256-VRC26 UCA (light gray) and the subsequent development of two distinct mAb sublineages. The sublineage with restricted evolution contains dead-end mAbs (dark gray) which cannot tolerate viral escape mutations. The continually evolving sublineage contains bNAbs (red)

and off-track mAbs (turquoise). While both bNAbs and off-track mAbs have high levels of SHM, only the former mature to recognise multiple immunotypes, and therefore acquire breadth.