Difficult-to-neutralize global HIV-1 isolates are neutralized by antibodies targeting open envelope conformations

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The HIV-1 envelope (Env) is the target for neutralizing antibodies and exists on the surface of virions in open or closed conformations. Difficult-to-neutralize viruses (tier 2) express Env in a closed conformation antigenic for broadly neutralizing antibodies (bnAbs) but not for third variable region (V3) antibodies. Here we show that select V3 macaque antibodies elicited by Env vaccination can neutralize 26% of otherwise tier 2 HIV-1 isolates in standardized virus panels. The V3 antibodies only bound to Env in its open conformation. Thus, Envs on tier 2 viruses sample a state where the V3 loop is not in its closed conformation position. Envelope second variable region length, glycosylation sites and V3 amino acids were signatures of neutralization sensitivity. This study determined that open conformations of Env with V3 exposed are present on a subset of otherwise neutralization-resistant virions, therefore neutralization of tier 2 HIV-1 does not always indicate bnAb induction.
The human immunodeficiency virus subtype 1 (HIV-1) envelope (Env) protein is a heterodimeric trimer consisting of gp120 and gp41 subunits. The gp120 is divided into five conserved (C1–C5) regions and five variable loop (V1–V5) regions based on primary amino acid sequence. The gp120 engages its receptor CD4, after which, it undergoes conformational changes that expose variable loop regions and the coreceptor binding site on an open Env conformation. There are many Env epitopes that confer antibody neutralization of HIV-1 with open Env conformations (tier 1 viruses), and during natural infection, antibodies targeting these epitopes dominate the antibody response. The third variable region (V3) is among the most immunogenic regions on Env, and is relatively conserved compared to other hypervariable regions. However, antibodies specific for the V3 region have not been broadly neutralizing, presumably because the V3 region is not accessible on HIV-1 Env prior to conformational changes induced by CD4 engagement. Unliganded HIV-1 Env structures showing a hidden V3 loop are consistent with this notion. Furthermore, this hypothesis is supported by the enhanced HIV-1 neutralization by V3-specific antibodies in the presence of soluble CD4. In the absence of CD4, mutations in the hydrophobic core of the gp120 subunit or at the N301 and N160 glycosylation sites can render the V3 region accessible for V3-specific neutralizing antibodies. Thus, amino acid sequence or glycosylation changes are sufficient for V3 region exposure on Env in the absence of CD4 binding.

HIV-1 Env is the sole target for HIV-1 broadly neutralizing antibodies (bnAbs). To understand the significance of neutralization of diverse HIV-1 isolates, HIV-1 isolates have been typed into tiers based on their sensitivity to polyclonal neutralizing antibodies in sera from HIV-1-infected individuals. Viruses have been typed as tier 1A, 1B, 2, or 3, with 1A being the most neutralization-sensitive tier 1 viruses, and during natural infection, antibodies targeting these epitopes dominate the antibody response. The third variable region (V3) is among the most immunogenic regions on Env, and is relatively conserved compared to other hypervariable regions. However, antibodies specific for the V3 region have not been broadly neutralizing, presumably because the V3 region is not accessible on HIV-1 Env prior to conformational changes induced by CD4 engagement. Unliganded HIV-1 Env structures showing a hidden V3 loop are consistent with this notion. Furthermore, this hypothesis is supported by the enhanced HIV-1 neutralization by V3-specific antibodies in the presence of soluble CD4. In the absence of CD4, mutations in the hydrophobic core of the gp120 subunit or at the N301 and N160 glycosylation sites can render the V3 region accessible for V3-specific neutralizing antibodies. Thus, amino acid sequence or glycosylation changes are sufficient for V3 region exposure on Env in the absence of CD4 binding.

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We tested the ability of these vaccine-elicited antibodies to neutralize tier 2 HIV-1 viruses in four standardized panels of viruses — multiclade 30-virus panel, early/acute clade C 100-virus panel (composed of 7 tier 1 and 93 tier 2 viruses), the global 12-virus panel, and a global 208-virus panel (Supplementary Fig. 2). There was overlap of viruses among the panels, such that there was a total of 292 unique tier 2 HIV-1 pseudoviruses tested. The antibody DH796.1 neutralized significantly more viruses (26% of the 292 viruses) than DH727.2 and DH753 as determined by IC50 titers (Fig. 1c, Supplementary Fig. 3A and Supplementary Data 1; Fisher’s exact test p = 0.005 for DH796.1 compared to DH753 and p = 0.003 when compared to DH727.2, n = 292). The neutralization breadth of DH796.1 decreased to 10% when neutralization was considered as inhibition of 80 percent of virus replication (IC80; Fig. 1d and Supplementary Data 1). Neutralization potency determined as geometric mean IC50 was modest for all three antibodies against all 292 viruses (DH796.1 = 2.6, DH727.2 = 4.6, DH753 = 2.1 μg/mL⁻¹), and among positive responses there were no significant differences in potency of the 3 antibodies (p > 0.05, Wilcoxon Rank sum test and Supplementary Fig. 1c, 2c, and 3a). None of the antibodies blocked CD4 binding to envelope, hence this was not the mechanism of neutralization (Supplementary Fig. 4).

In some cases, antibodies can neutralize a substantial proportion of the replicating virus, but do not eliminate all HIV-1 replication. As a measure of complete neutralization we examined the maximum neutralization achieved against the tier 2 clade C early/acute viruses. For the 93 tier 2 viruses in this panel, incomplete neutralization was most evident for DH753, where 18 tier 2 early/acute clade C viruses were neutralized greater than 50% but none of the viruses were neutralized above 90% (Fig. 1e). DH796.1 and DH727.2 inhibited virus infection by 90% or greater for only two and one virus respectively (Fig. 1e). Thus, these antibodies lacked complete neutralization against most early/acute clade C viruses tier 2 HIV-1 isolates.

Although viruses are categorized into tiers, the sensitivity of the viruses within a tier still varies creating a spectrum of antibody neutralization sensitivity. We examined their neutralization potency against 101 tier 2 viruses with known sensitivities to 205 HIV-1-infected polyclonal sera. The 101 tier 2 viruses demonstrated a range of sensitivities to the HIV-1-infected polyclonal sera, which allowed them to be ranked according to differences in the geometric mean inhibitory dilution 50 (ID50) titer for all 205 plasma samples (Fig. 1f and Supplementary Data 2). The tier 2 viruses with detectable IC50s for DH727.2,
DH753, or DH796.1 tended to be at the most sensitive end of the neutralization spectrum (Fig. 1f). There were 21 viruses with detectable IC50 values and 18 of these 21 isolates were at the most sensitive end of the tier 2 virus spectrum (Fig. 1f). There were 21 viruses with detectable IC50 values and 18 of these 21 isolates were at the most sensitive end of the tier 2 virus spectrum (Fig. 1f). Therefore, the tier 2 viruses that were resistant to the vaccine induced V3 antibodies (Fig. 1f) and Supplementary Data 2). Therefore, the tier 2 viruses that were resistant to the vaccine induced V3 antibodies (Fig. 1f) and Supplementary Data 2). Thus, tier 2 isolates possessed distinct susceptibility to V3 antibody neutralization based on which end of the neutralization sensitivity spectrum they resided.

BG505 SOSIP trimer immunization has been reported to elicit serum neutralization activity in macaques against X1632-S2-B10, Ce1176_A3, 25710-2.43, and 398-F1_F6_20 in the 12-virus global panel [29]. We assessed whether our three macaque neutralizing antibodies could neutralize the same isolates in the 12-virus panel. X1632-S2-B10, CNE8, and 398-F1_F6_20 were sensitive to 3074, a human V3 antibody from natural infection (Supplementary Fig. 3b). Although none of the antibodies potently neutralized these viruses, three HIV-1 isolates (398-F1_F6_20, 25710-2.43, and X1632-S2-B10) were found to be neutralized by both DH796.1 and BG505 induced macaque sera [29] (Fig. 1h). DH796.1 differed from the BG505 SOSIP immune sera...
in that it did not neutralize Ce176_A3, but instead neutralized CNE8, BJ0X002000.03, and CH119.10. Thus, 3 of 4 viruses sensitive to BG505 SOSIP immune sera were also sensitive to our vaccine-elicited macaque V3 antibodies.

HIV-1 clade-specific antibody neutralization activity. We examined differences in antibody neutralization across HIV-1 clades for the 208 viruses in the large global panel (Supplementary Fig. 2a). DH753 neutralized a subset of clade B viruses, while clade B viruses were almost completely resistant to DH727.2 and DH796.1 (Fig. 2). Conversely, clade C viruses were neutralized by all 3 antibodies (Fig. 2). Some clades were rarely neutralized, or only weakly, by any of the 3 antibodies, including clades A and D and the circulating recombinant form CRF01 (Fig. 2). Clade preferences were also evident for two antibodies with moderate neutralization breadth from infection (447-52D and 3074). The antibodies tended to show a preference for the clade of the infecting or vaccine virus that stimulated the response. 447-52D, isolated from a person with a clade B infection, was Specific for clade B viruses—driven by the clade B GPGR motif that is common only in clade B (Fig. 2). Antibody 3074, isolated from a person with a Circulating Recombinant Form 02 (CRF02) infection (an AG recombinant which is mostly clade A in Env), had relatively strong CRF02 responses, as well as additional responses. DH727.2 elicited by a clade C vaccine, CH505, favored...
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V3 antibody HIV-1 neutralization signatures. To determine the virus attributes that contributed to V3 antibody neutralization sensitivity we examined the sequences of the more than 200 viruses tested for neutralization. First, we compared IC50 neutralization titer with length of, net charge of, and number of glycosylation sites within V1, V2, and V1 + V2 combined hypervariable regions. The V1 and V2 loop region was selected because of structural evidence showing that these loops may block access to the V3 loop when Env is in a closed conformation. We divided the viruses into two groups, those with V2 lengths above the median and those at the median or below (the V2 length range was between 3 and 20, and the median was 8). Next, we used a Fisher’s exact test to compare the frequency of positive IC50 values in each group. 447-52D and DH753 neutralization showed a significant association with V2 length ($p < 0.006$ with Bonferroni correction), where pseudoviruses with long V2 loops were significantly more likely to be resistant (Fig. 4a). 3074 showed a supporting trend, and DH796.1 and DH727 were less well-powered and not significant (Fig. 4a). There was a weak trend suggesting that a few glycosylation sites in the V2 hyper-variable region may also be associated with sensitivity ($p$-values of 0.01).

V3 region signatures patterns were specific for particular antibodies (Fig. 4b). Within the V3 loop, amino acids at positions 308, 315, 316 were associated with sensitivity to different antibodies. For example, Arg308 was required for DH796.1 and DH727 neutralization activity consistent with its requirement for binding in peptide microarrays (Figs. 3b and 4b). In contrast, Arg308 was associated with 447–52D resistance (Fig. 4b). In contrast, Arg315 was favored for 447–52D sensitivity, preferred for DH753 sensitivity, and most common in clade B envelope sequences (72%). Gln315 was most common in other clades and was strongly favored by both DH796.1 and DH727.2 (Fig. 4b). Thus, these signatures may be driving the distinctive clade specificities of these antibodies (Fig. 2). Potential N-linked glycosylation sites (PNGS) at the base of the V3 loop at positions 295 and 332 were enriched among viruses sensitive to DH753 and 447–52D. These glycosylation sites were located proximal to the Cys that form the disulfide bridge that closes the V3 loop, and may impact its orientation in an intact protein. A PNGS at position 230 was also enriched for 447–52D.

Structural analysis of antibody binding to Env V3 peptides. We determined the crystal structure of two of the vaccine-induced antibodies bound to V3 peptides to determine the roles of amino acids identified in the neutralization signature analysis. First, we solved the crystal structure of DH753 in complex with ZAM18 (NNTRKSKIIRPGQAFYATGGIIG), a sequence closely resembling clade A and C virus sequences (Fig. 5a and Supplementary Fig. 5a). Second, we solved DH753 bound to a clade B MN V3 peptide (YNKRRHIIHGPRAYTTYKNIIG; Supplementary Fig. 5c). The two peptides were chosen because their structures in complex with antibody 3074 have been previously solved.

Structures of the complexes were determined to 2.2 and 2.7 Å resolution, respectively (Supplementary Table 2). The DH753:ZAM18 structure showed electron density for residues 305–320 and the MN complex structure showed electron density for residues 306–321. Both DH753 structures showed the V3 loop peptides bound in the same orientation with an RMSD of 0.86 Å when the DH753 Fv regions were aligned (Supplementary Fig. 5c). DH727.2 Fab was crystallized in complex with ZAM18 V3 peptide (gp120$_{301-325}$) to 1.8 Å resolution (Fig. 5b, Supplementary Fig. 5b). Electron density of the V3 peptide was sufficient to build 14 residues comprising the central segment (residues 305–318) of the peptide (Fig. 5b). The peptide adopted the $\beta$-hairpin conformation typical of similar polypeptides representing the V3 crown.

Side- and crown-approach modes of V3 peptide recognition. Structural analysis of liganded-DH727 confirmed that DH727.2 had an overlapping epitope with V3 Ab 3074 (Fig. 5c). Also, DH727.2 showed similarity to other V3 peptide antibodies in that its paratope exhibited a defined cleft between the heavy and light chains into which the $\beta$-hairpin structure of the V3 crown bound.

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-10899-2 | www.nature.com/naturecommunications
oblique approach to the V3 crown β-hairpin conformation (Fig. 5c). In contrast, DH753 bound the V3 β-hairpin head-on at its apex (the β-turn itself) (Fig. 5a, c). A side approach mode of binding is more frequently observed among V3 antibodies; however, there are other examples of crown approach antibodies as well (Fig. 5c). DH753 showed a rare, exceptionally deep binding pocket on its paratope (Fig. 5d), which has only been observed for one other V3 antibody, 537–10D39.

Primary interactions between antibody DH727.2 and antigen were hydrogen (H)-bonds between the terminal amides of the V3 Arg308 side chain with the side chain hydroxyl and backbone carbonyl of Thr97 on the light chain. H-bonds were also observed with the side chain of V3 residue Gln315 and both heavy chain residue Asn59 and light chain residue Thr100. These interactions provided a structural explanation for why Arg308 and Gln315 were found to be a virus sequence signature and were important for peptide binding (Figs. 3b and 4b). Lastly, favorable H-bonding was also observed between the backbone carbonyl of V3 residue Ile309 and the side chain of Arg99 in HCDR3.

In complex with ZAM18 peptide, the binding interface between DH753 and V3 included a dual salt bridge between the side chains of heavy chain residue Asp53 and V3 residue Arg308 (Fig. 5e). Thus, Arg308 was a common contact residue for DH727.2 and DH753. Additionally, contacts between antibody and the V3 polypeptide backbone occurred between the side chain of HCDR3 residue Tyr100B and V3 residue Ile309 as well as heavy chain residue Trp47 with V3 residue Pro313 (in the GPGx arch). In complex with MN peptide,
DH753 could not establish the above noted salt bridge between the heavy chain Asp53 and V3 Arg308 side chains since ZAM18 possessed a His at position 308 (Fig. 5e). However, an intermediary phosphate polyatomic ion mediated a polar interaction between the two side chains. This phenomenon was present in both Fab-peptide complexes in the asymmetric unit. The LCDR3 residue Trp91 had van der Waals contacts with Gln315 in the ZAM18 peptide and Arg315 in the MN peptide, explaining why polymorphisms were tolerated at that position (Fig. 5e).

**Molecular modeling of V3 antibodies bound to trimeric Env.**

To model potential antibody:Env interactions, we superimposed the Fab:peptide structures onto several Env models by aligning the V3 peptide to the Env, and allowing this alignment to determine the hypothetical orientation of the Fab. When the DH727 structure was aligned onto a single gp120 of a closed envelope trimer there was unrealistic overlap (Fig. 6a), giving 102 clashes between backbone atoms of the Fab heavy chain and the V1V2 domain. DH753 showed 150 backbone clashes that involved the Fab light chain overlapping the V1V2 domain and
the heavy chain overlapping the region below the V3 loop (Fig. 6b). These observed clashes between Fab and the V1V2 domain may explain why short V1V2 regions were determined to be a signature of viruses sensitive to V3 antibody neutralization (Fig. 4a). Thus, Fab binding seemed to require opening of the trimer, which would rearrange the V3 loop and/or the V1V2 domain. In agreement with this notion, in the CD4-induced structure of gp120, the V3 loop projects away from the gp120 core and would be freely available to bind DH727.2 or DH753 Fab (Fig. 6c–d). When modeled onto a fully open trimer either Fab would be able to bind freely without clash (Fig. 6e–h).

V3 neutralizing antibodies lack binding to closed Env. Soluble CD4 preincubation experiments have suggested conformational masking of the V3 loop precludes antibody binding to Env12. We determined the role of conformational masking in DH727.2, DH753, and DH796.1 binding to recombinant gp120, unstabilized SOSIP.664 gp140, and stabilized DS.SOSIP.664 gp14013,42,43. The unstabilized SOSIP trimer is recognized by 19B and 17B and thus samples the CD4-triggered state of the trimer43. In contrast, the CH505 TF DS.SOSIP would be more similar to a pre-CD4 bound Env as it is devoid of binding by CD4-induced antibody 17B42. Moreover, the DS.SOSIP possesses a disulfide bond between Cys201C and Cys433 that prevents conformational transitions to the open conformation13. Each envelope was derived from the CH505 transmitted/founder virus, therefore, their V3 loop sequences were identical (Fig. 7a). Each antibody bound to the CH505 TF gp120 (Fig. 7b, first column). Presentation of the V3 loop in the context of an Env trimer reduced DH753 binding to below detectable levels. DH796.1 and DH772.2 were able to engage the unstabilized trimer that frequently samples the open conformation, although association with trimer was slower than with the gp120 (Fig. 7b, second column). For DH772.7 and DH796.1 the dissociation from trimeric Env was slower than monomeric gp120 resulting in overall improved binding affinities of 3.5 and 10.8 nM respectively (Fig. 7c). When the trimer was stabilized in the presumably pre-CD4 triggered state13 none of the V3 antibodies were able to engage the Env to detectable levels. Thus, conformational fixation of the pre-CD4 bound state of the Env trimer rendered the V3 region inaccessible by the three rhesus macaque antibodies, showing that for these three V3 antibodies, Env conformation dictated antibody binding to CH505 TF Env trimer.

To further explore the Env trimer conformational recognition by DH772.2, DH753, and DH796.1 we triggered envelope into the open state by the addition of CD4 and measured antibody binding to the envelope.13,44. In contrast to binding affinity experiments, saturating concentrations of antibody were coated on SPR sensor chips to allow avidity to contribute to binding. Inducing the unstabilized into the CD4-induced open state increased binding by the V3 antibodies (Fig. 7d). The increase in binding suggested that the unstabilized trimer possessed some molecules that were not in the fully open conformation at equilibrium. Binding to the stabilized, conformationally-fixed trimer was low and the addition of CD4 did not improve binding to this Env (Fig. 7e13). Taken together, the antibodies bound optimally to Env trimers that were capable of sampling multiple states, including the open state, but poorly recognized trimers that did not transition from the closed state.

Ontogeny of tier 2 neutralizing V3 antibodies. We investigated the ontogeny of the V3 antibodies from vaccination by isolating two additional members of the DH727 lineage and 5 additional DH796 members (Supplementary Table 1). A maximum likelihood phylogenetic tree was constructed to infer the unmutated common ancestor (UCA) and intermediate antibodies of the DH727 and DH796 clonal lineages (Supplementary Fig. 6a). Of the three antibody lineages only the DH772 UCA was capable of binding HIV-1 envelope (Supplementary Fig. 6b). The DH727 UCA bound to the vaccine immunogen CH505 TF gp120 and V3 peptides from clade C consensus (Con C) and CON-S viruses. For the DH727 and DH796 antibody lineages, the somatically mutated early intermediate antibodies in the lineages bound with higher titers than the UCA (Supplementary Fig. 6b). The DH727 UCA neutralized CNER (IC50 = 0.43 μg mL1) as did all other members of the DH727 lineage (Supplementary Table 1; Supplementary Fig. 6c). The minimally mutated lineage members also showed weak neutralization of tier 2 virus 25710-2.43 (IC50 range = 6–30 μg mL1) (Supplementary Table 1; Supplementary Fig. 6c). The DH753 UCA and DH796 UCA did not neutralize any of the 6 viruses assessed, but early DH796 intermediate antibodies neutralized 3 of 6 viruses examined (Supplementary Fig. 6c). Therefore, neutralization activity arose with only little somatic mutation.

Discussion
Here we demonstrated that Env V3 loop antibodies induced by vaccination can neutralize HIV-1 primary isolates categorized as tier 2, difficult-to-neutralize viruses. Studies have shown that V3 region antibodies could neutralize select primary isolates, but lacked detectable breadth9,31,45–47. In this study we performed a comprehensive assessment of the neutralization breadth of V3 region antibodies on large standardized panels of 292 HIV-1 viruses to definitively show the neutralization potential of tier 2 virus-neutralizing V3 region-specific antibodies. These large virus panels are the gold standard for determining HIV-1 neutralization breadth, and are used to signal the presence of bnAbs or their precursors across vaccine trials and infected human
samples\textsuperscript{19,26,27}. However, we determined that weak neutralization of viruses within these standardized panels can be due to V3 antibodies—not antibodies against the known broadly neutralizing epitopes on tier 2 HIV-1 Envs with closed conformations.

Recent vaccination studies have reported weak or moderate neutralization of tier 2 HIV-1 strains\textsuperscript{45,47,48}. Comparing a recent report of inducing tier 2 neutralizing antibodies in macaques to the isolates neutralized by the V3 antibodies in this study, 3 of 4 viruses were sensitive to the V3 antibodies\textsuperscript{29}. Thus, when low titer neutralization is observed in polyclonal sera care must be taken to rule out V3-specific neutralizing antibodies as the mediators of the tier 2 or primary isolate neutralization. Also, it should be noted that not all HIV-1 V3 antibodies possess the same neutralization breadth, and the breadth can be highly clade dependent (Fig. 2). In our combined viral panels, DH796.1 was more potent and broader than the other vaccine-induced V3 antibodies as well as the best-in-class V3 antibodies from human infection 3074, and 447-52D (Supplementary Data 1). Therefore, a particular isolate may be resistant to several known V3 monoclonal antibodies, but it should not be assumed that infection-induced or vaccine-elicited polyclonal sera do not contain V3 antibodies capable of neutralizing those viruses. The complexities of

**Fig. 5** Structural analysis of macaque V3 antibodies. 

- **a** The crystal structure of DH753 is shown with the L chain rendered in lilac, the FabH chain in dark purple, and bound ZAM18 V3 hairpin peptide in yellow. 
- **b** The crystal structure of DH727.2 is shown in a similar orientation with the L chain in salmon, the FabH chain in red, and the ZAM18 V3 peptide again in yellow. 
- **c** A superposition of several V3 antibody-peptide structures on the basis of the peptide (yellow) showed two clusterings. The ‘crown approach’ cluster included DH753 and other antibodies depicted in purple shades. The ‘side approach’ cluster included DH727.2 and other antibodies depicted in red shades. 
- **d** DH753 bound the V3 hairpin head-on by virtue of a deep pocket in its paratope. 
- **e** Structures of DH753 with two different V3 peptides were determined in order to scrutinize requirements for interaction. Some conservative mutations are tolerated in the antibody-antigen interface. For instance, mutation of the long Arg308 side chain to the shorter His became a through-interaction mediated by a phosphate ion.
interpreting the low neutralization titers from sera or plasma highlight the importance of identifying the monoclonal antibodies that mediate the neutralization activity, or at the very least performing definitive plasma neutralization mapping experiments.

HIV-1 neutralization by these newly isolated V3 antibodies often did not reach 100 percent. This incomplete neutralization has been observed in the past for glycans-dependent antibodies28, which may be due to differences among Env glycosylation profiles. The underlying mechanisms for incomplete neutralization by V3 region antibodies are speculative at present, but may be related to Env structural dynamics21,49 or glycosylation changes15. Amino acid sequence is clearly important as our signature for each antibody. Additionally, the Env structure was important for recognition as well. In surface plasmon resonance experiments, Env trimer conformations antigenically resembling the CD4-induced state were bound by V3 antibodies with high magnitude, whereas soluble trimers stabilized in a non-CD4-induced state were bound by V3 antibodies with high preference. Amino acid sequence is clearly important as our signature for each antibody. Additionally, the Env structure was important for recognition as well. In surface plasmon resonance experiments, Env trimer conformations antigenically resembling the CD4-induced state were bound by V3 antibodies with high magnitude, whereas soluble trimers stabilized in a non-CD4-induced state were bound by V3 antibodies with high preference. Amino acid sequence is clearly important as our signature for each antibody. Additionally, the Env structure was important for recognition as well. In surface plasmon resonance experiments, Env trimer conformations antigenically resembling the CD4-induced state.
Crystallography. Fabs were expressed in transiently-transfected Expi293 cells (Invitrogen, Cat No. A14527). The Fab was purified by KappaSelect affinity chromatography. The Fab was eluted off of the KappaSelect resin with a glycine pH 2.4 buffer and the pH was subsequently neutralized by the addition of Tris pH 8.0. The Fab was then further purified via size exclusion chromatography. Peak protein-containing fractions were concentrated, buffer exchanged to ddH2O, and brought to 15.0 mg ml\(^{-1}\). Fabs were mixed with V3 peptides corresponding to gp120 in a 1:3 molar ratio. The V3 peptides were replicated from previously published V3 crown antibody structures. The complexes were tested against commercially available screens (Qiagen, Molecular Dimensions) in SBS format sitting drop plates via automation (Douglas Instruments Ltd) with 60 \(\mu\)l reagent reservoirs and drops composed of 0.2\(\mu\)l protein with 0.2\(\mu\)l reservoir. Crystals for DH727.2 with ZAM18 peptide were observed over a reservoir of 0.1 M Tris pH 8.5, 20% PEG 6000. Crystals for DH753 with ZAM18 peptide were observed against a reservoir of 0.2 M sodium sulfate decahydrate, 20% PEG 3350. Crystals for DH753 with MN peptide were observed against a reservoir of 0.2 M ammonium dihydrogen phosphate, 20% PEG 3350. All crystals were briefly soaked in reservoir supplemented with ~20% ethylene glycol then flash-frozen in liquid nitrogen.

Data were collected at Southeast Regional Collaborative Access Team (SER-CAT) 22-ID (or 22-BM) beamline at the Advanced Photon Source, Argonne National Laboratory. Diffraction data for all crystals were collected at SER-CAT with an incident beam of 1 \AA{} in wavelength. The DH727.1 + ZAM18 structure
Fig. 7 Closed Env conformation limits recognition of the V3 loop by tier 2 neutralizing, vaccine-induced antibodies. a CH505 TF gp120 and SOSIP trimer V3 amino acid sequence alignment. Amino acids identical to CH505 gp120 are shown as dots. b Surface plasmon resonance sensograms of antibody binding to serial dilutions of CH505 TF gp120, un­stabilized SOSIP trimer that transitions to the open conformation, and SOSIP trimer stabilized in the closed conformation. Each row shows binding by an individual antibody. Each column shows binding to the specified version of HIV-1 Env. c Apparent equilibrium dissociation constants (K<sub>d</sub>) for each antibody binding to the different forms of rec­ombinant Env shown in b. Antibodies with binding magnitudes too weak to measure a K<sub>d</sub> are listed as no binding. d, e Induction of trimer opening by soluble CD4 antibodies binding to unstabilized trimers but not con­formationally fixed, stabilized trimers. Surface plasmon resonance binding of rhesus monoclonal antibodies DH727.2, DH735, and DH796.1 to CH505 TF Env in the absence (open bar) or presence (filled bar) of soluble CD4. Antibody binding to d unstabilized CH505 TF trimer and e stabilized CH505 TF trimer is shown. The stabilized Env trimer (DS.SOSIP) contains the 201C-433C disulfide bond that prevents CD4 triggering. Symbols indicate independent measurements, and the bars represent the mean of the duplicate independent experiments. Source data are provided as a Source Data file.

showed 96.8% of amino acid residues in favored areas of the Ramachandran plot with none in outlying regions. The DH753 + ZAM18 structure similarly showed 97.0% of residues in favored regions with no outliers. The DH753 + MN structure had 93.1% of residues in favored regions with just 0.8% in outlying regions. Data were refined in HKL-2000. The DH727.2-ZAM18 peptide structure was phas­ed by molecular replacement in PHENIX using as the search model the Fab fragment of an antibody to Madcam-1 D2d as selected by high sequence identity. Likewise, the DH753-ZAM18 structure was phased using a complex search model generated from the Fab fragment of the HIV antibody 10E8 germline heavy chain (PDB: 3MGW) and the HIV-1 D01 light chain (PDB: 1X3D). The D01 light chain was in turned using the DH753-ZAM18 structure as the search model. Rebuilding and real-space refinements were performed in Coot with reciprocal space refinements in PHENIX and validations in MolProbity.

Molecular modeling of antibody binding to HIV-1 trimer. All modeling was done using UCSF Chimera. The Fab model of the Fab interactions with the closed gp120, we used chain G from a BG505 SOSIP structure (PDB: 4ZMJ[https://doi.org/10.2210/pdb4ZMJ/pdb]), and docked the Fab-peptide structures reported here onto the closed gp120, using only the peptide fragment of the Fab structure for alignment onto the gp120. Clashes between the gp120 and the Fab were calculated with Chimera’s Find Clashes/Contacts function, using the default clash settings and specifying only backbone atoms. For the open monomer and trimer, we built homology models for the CH505 TF SOSIP sequence using the open BG505 structure (PDB: 5VN3[10.2210/pdb5VN3/pdb]) as a template for SWISS-MODEL, and docked the Fab as above.

HIV-1 Env peptide array. The HIV-1 peptide libraries contain overlapping HIV-1 peptides covering full-length gp120 of 5 consensus viruses from group M and clades A, B, C, and D. Array slides were provided by JPT Peptide Technologies GmbH (Germany) by printing a library of peptides onto epoxy glass slides (PolyAyn GmbH, Germany). The library contains overlapping peptides (15-mers overlapping by 12) covering 5 full-length gp160 consensus sequences (clade A, B, C, D, and group M) and 10 consensus sequences (15-mers overlapping by 12) for 7 consensus sequences (clade A, B, C, D, CRF1, CRF2, and group M) and 6 vaccine strains (MN, A244, TH023, TV-1, ZM651, 1086 C). Three identical subarrays were blocked for 1 h, followed by a 2-h incubation with monoclonal antibody, and a subsequent 45-min incubation with anti­mouse IgG-conjugated to AP647 (Jackson Immunolaboratories, PA). Array slides were scanned at a wavelength of 635 nm using an InnoScan 710 scanner (Innopsy, Denmark) and images were analyzed using Magpix V8.1.1.

Antigen-specific single B cell sorting. Cryopreserved PBMC were washed and counted. The PBMC was stained with NK, T, and B cell surface markers and fluorophore-labeled envelope protein for 1 h at 4 °C. Antibodies used for staining were CD20 PE/Cy7 clone L27 (BD Biosciences Cat No. 347673), CD3 PerCP Cy5.5 clone 145-2C11 clone L27 (BD Biosciences Cat No. 552852), and CD7 PE/Cy7 clone 69B.3 clone L27 (BD Biosciences Cat No. 359774). The 69B.3 antibody was analyzed for a library of 16 gp120 variants.

Monoclonal antibody competition ELISAs. Nuncscop plates were coated with HIV-1 envelope, washed and blocked with Superblock. After blocking was complete, non-biotinylated monoclonal anti­bodies were serially diluted in SuperBlock starting at 100 μg mL<sup>−1</sup> and incubated in triplicate wells for 90 min. To determine relative binding no antibody was added to a group of wells scattered throughout the plate. After 90 min the non-biotinylated antibody was washed away and biotinylated monoclonal antibodies or soluble CD4 was incubated in the wells for 1 h at sub-saturating concentrations. The binding of CD4 was detected with biotinylated anti-CD4 antibody OKT4. As a positive control, the same antibody was used to block itself. For CD4 blocking assays the CD4 binding site antibody CH106 was used as positive control antibody. As a negative control an antibody or soluble CD4 was tested. Antibody binding was compared to the presence of competing antibody to calculate percent inhibition of binding. Based on historical negative controls, assays were considered valid if the positive control antibodies blocked greater than 20% of the biotinylated antibody binding.

In vitro HIV-1 neutralization. Antibody-mediated HIV-1 neutralization was measured using Tat-regulated luciferase (Luc) reporter gene expression to quantify reductions in virus replication in TZM-bl cells. TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program, as contributed by John Kappes and Xiaoyun Wu. The monoclonal antibody was pre-incubated with virus (~150,000 relative light unit equivalents) for 1 h at 37 °C, and TZM-bl cells were subsequently added. After 48 h cells were lysed and luc activity determined using a microtiter plate luminometer and BriteLite Plus Reagent (Perkin Elmer).

Neutralization titers are the inhibitory concentration at which relative luminescence units (RU) were reduced by 50% or 80% compared to RU in virus control wells after subtraction of background RU in cell control wells (IC50 and IC80 respectively).

Recombinant antibody production. Expi293 cells (Invitrogen, Cat No. A14527) were cultured in Expi293 media at less than 5 × 10<sup>6</sup> cells mL<sup>−1</sup>. On the day of transfection cells were diluted to a final volume of 0.5 L at a concentration of 2.5 × 10<sup>6</sup> cells mL<sup>−1</sup> in Expi293 media. The 293 cells were co-transfected with 400 μg of heavy chain plasmid and light chain plasmid using ExpertFectamine. Five days after transfection cell culture media was cleared of cells by centrifugation and 0.8 M filtration. The cell-free supernatant containing IgG1 was incubated with protein A resin (ThermoFisher) overnight at 4 °C. The protein A resin was centrifuged and cell culture supernatant was removed. The resin was washed with 5 mL of PBS with 340 mM NaCl. Thirty mL of 10 mM glycine pH 2.4, 150 mM NaCl were used to elute the antibody off of the affinity resin. The pH of the eluted antibody solution was increased to neutral pH by adding 1 M Tris pH 8.0. The neutral pH eluate was buffer exchanged into PBS with sequential rounds of centrifugation, filtered, and stored at −80 °C.

Recombinant SOSIP Env production. Freestyle 293 (Invitrogen, Cat No. R79007) cells were cultured in Freestyle 293 media below 3 × 10<sup>6</sup> cells mL<sup>−1</sup>. On the day of transfection, cells were diluted to 1.25 × 10<sup>6</sup> cells mL<sup>−1</sup> with fresh media and 1 L of cells was transfected with 293Fectin (Life Technologies) and 650 μg of SOSIP plasmid expressing plasmid DNA respectively. Cells were cultured for 6 days at 37 °C and 5% CO<sub>2</sub> in an incubator humidified incubator. At the end of the transfection, cell cultures were centrifuged for 30 min at 3500 rpm and 80μm
filtered. The cell-free supernatant was concentrated to less than 100 mL with a single-use tangential flow filtration cassette and 0.8 μm filtered again. Trimeric Env protein was purified with PCT145 affinity chromatography. One hundred mg of PGT145 IgG1 antibody was conjugated to 10 mL of CNBr-activated sepharose (GE Healthcare). Coupled resin was packed into Tricorn column (GE Healthcare), and stored in PBS supplemented with 0.05% sodium azide. Cell-free supernatant was applied to the column at 2 mL min⁻¹ using a AKTA Pure (GE Healthcare). The column was washed with 20 mL of column buffer, and protein eluent was eluted off of the column with 3M MgCl₂. The eluate was immediately diluted in 10 mM Tris pH8, 0.2 μm filtered, and concentrated down to 2 mL for size exclusion chromatography. Size exclusion chromatography was performed with a Superose 16/600 column in 10 mM Tris pH8, 500 mM NaCl. Fractions containing trimeric HIV-1 Env protein were pooled together, sterile-filtered, snap frozen, and stored at −80 °C. Recombinant Env gp120 production. Recombinant gp120 was expressed in Freestyle 293 cells (Invitrogen, Cat No. R79007) 24. Cells were transfected with PEI:DNA complexes, and cultured for 5 days. Recombinant protein was purified with Galanthus nivalis lectin-agarose (Vector Laboratories), buffer exchanged into phosphate buffered saline and stored at −80 °C. Phylogenetic Trees. Maximum likelihood trees were generated using PhyML67 with the HIVb model (https://www.hiv.lanl.gov/content/sequence/PHYML/ interface.html), and represented using Rainbow Tree at the Los Alamos database (https://www.hiv.lanl.gov/content/sequence/RAINBOWTREE/rainbowtree.html).

Neutralization signature analysis. We performed a phylogenetically corrected analysis with a liberal threshold of q = 0.3 for an inclusive sweep of the full protein alignment. We next used a simple signature analysis, with no phylogenetic correction, to identify potential signatures associated with neutralization sensitivity within the V3 loop region, including the PNGS sites that bound it on either side at N295 and N332. Because these sites are near the antibody contact regions, they are good candidates for direct involvement in antibody interactions, and may be responsible for the antigenic phenotype of the Env as well as for the clade specificity of V3 antibodies. But without the phylogenetic correction it is also possible that these signatures may be carried along with the site or sites that more directly determine the positive/negative sensitivity phenotype. For example, Arg315 is required for 447-52D neutralizing activity and it is highly enriched in the B clade relative to other clades. Other amino acids that are specifically enriched in the B clade may also be associated with 447-52D, that are not directly impacting the phenotype but are genetically linked to Arg315. The LOGOS of the signature sites were created using the Los Alamos database Analyze Align tool (https://www.hiv.lanl.gov/content/sequence/ANALYZEALIGN/analyze_align.html).

Direct ELISA. In total 2 μg mL⁻¹ of protein in sodium bicarbonate buffer was incubated in sealed Nunc-absorb (ThermoFisher) plates overnight at 4 °C.24 Unbound protein was washed away and the plates were blocked with SuperBlock for 1 h. Serially dilution of monoclonal antibodies were added to the plate for 90 min. Binding antibodies were detected with 1:30,000 dilution of HRP labeled anti-IgG Fc antibodies (Southern Biotech, SB108a, Cat no. 4700-05). HRP was detected with 3,3′-5,5′-Tetramethylbenzidine. Binding titers were analyzed as area-under-the curve of the log-transformed concentrations.

Surface plasma resonance (SPR). SPR experiments were performed on a BIACore T200. For kinetics measurements approximately 50 RU of each antibody was captured on an anti-human IgFc immobilized Series S CM5 sensor chip (GE Healthcare) using an AKTA Pure (GE Healthcare) column. Serial dilutions of HIV-1 Env was flowed over the chip at 5 μL min⁻¹ using a Biocytin-buffered saline. The Env proteins were CH505 transmitted founder gp120, unstabilized chimeric 6.RSOSIP.664 gp140, and stabilized chimeric 6.RD.SOSIP.664 gp140. The stabilized chimeric SOSIP contains I201C and A433C. The stabilized chimeric SOSIP contains I201C and A433C and was captured on an anti-human IgFc immobilized Series S CM5 sensor chip (GE Healthcare). The code was further developed, and the interface for the code called GenSig, made available at the HIV database at Los Alamos (https://www.hiv.lanl.gov/content/GENETICSIGNATURES/gs.html) in conjunction with the publication by Bricat and colleagues69.

Data availability

Received: 6 January 2019 Accepted: 3 June 2019

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Acknowledgements
We acknowledge technical assistance from Giovanna Hernandez, Erika Dunford, Haiyan Chen, Esther Lee, Kedamawit Tilahun, Andrew Foulger, Aja Sanzone, Callie Vivian, Stormi Chadwick, Maggie Barr, Lawrence Armand, Elizabeth Carter, Kara Anasti, Tam Huynh and Arthur McMillan. We also thank the Duke Human Vaccine Institute Flow Cytometry core and Biomolecular Interaction Analysis Facility. Crystallography was performed in the Duke University X-ray Crystallography Shared Resource. SER-CAT is supported by its member institutions (see www.ser-cat.org/members.html), and equipment grants (S10 RR25528 and S10 RR028976) from the National Institutes of Health. Use of the Advanced Photon Source was supported by the U. S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38. This work was supported by NIAID extramural project grant R01 AI120801 (K.O.S.), and NIH, NIAID, Division of AIDS UMI grant AI100645 for the Center for HIV/AIDS Vaccine Immunology-Immunogen Discovery (CHAVI-ID; B.F.H.), NIH NIAID Duke Center for AIDS Research grant P30 AI064518 (S.X.S., G.D.T.), NIAID intramural research program (J.R.M.). The funders had no role in data collection and interpretation, or the decision to submit the work for publication.

Author contributions
Experimental Design; K.O.S., Q.H., N.I.N., and B.F.H.; Investigation and assays; Q.H., J.A.J., N.I.N., R.K.R., M.L., R.J.E., K.M., S.X.S., G.D.T., D.C.M., J.R.M., M.S., B.T.K., B.F.H., K.O.S.; Wrote manuscript K.O.S., R.J.E., N.I.N., R.K.R., M.L., S.M.A., R.J.E., B.T.K., S.X.S., G.D.T., D.C.M., J.R.M., M.S., B.F.H.; Funding: J.R.M., G.D.T., K.O.S., and B.F.H.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-10899-2.

Competing interests: B.F.H. and K.O.S. have patent applications submitted on EnvS used in this study. The remaining authors declare no competing interests.

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Peer review information: Nature Communications thanks James Paulson and other anonymous reviewer(s) for their contribution to the peer review of this work

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