Structural basis for llama nanobody recognition and neutralization of HIV-1

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SUMMARY

Nanobodies can achieve remarkable neutralization of genetically diverse pathogens including HIV-1. To gain insight into their recognition, we determined crystal structures of four llama nanobodies (J3, A12, C8, and D7), all of which targeted the CD4-binding site, in complex with HIV-1 envelope (Env) gp120 core, and also determined a cryo-EM structure of J3 with Env trimer. Crystal and cryo-EM structures of J3 complexes revealed this nanobody to mimic binding to the prefusion-closed trimer for both the primary site of CD4 recognition as well as a secondary quaternary site. In contrast, crystal structures of A12, C8, and D7 with gp120 revealed epitopes that included portions of the gp120-inner domain, inaccessible on the prefusion-closed trimer. Overall, these structures explain the broad and potent neutralization of J3 and the limited neutralization of A12, C8, and D7, which utilized binding modes incompatible with the neutralization-targeted prefusion-closed conformation of Env.

Keywords: CD4-binding site, cryo-EM, crystal structure, envelope trimer, HIV, llama VHH, nanobody, neutralization, single-domain antibody, steric clash

Introduction

To mount a successful infection, many viral pathogens, including influenza A viruses, beta-coronaviruses, and human immunodeficiency virus type 1 (HIV-1), engage host receptors through conserved receptor-binding surfaces. These conserved surfaces are often targeted by the humoral immune response, and many viral pathogens have adopted various means to evade these responses. In the case of HIV-1, the binding site for the primary human receptor, CD4, is surrounded by variable regions, recessed within a canyon-like depression, and shielded by glycans and conformational masking (Kwong et al., 2002; Kwong et al., 1998; Lee et al., 2016; Ozorowski et al., 2017; Stewart-Jones et al., 2016). These evasion mechanisms must allow access to the CD4-binding site (CD4bs) by the membrane-distal domain of CD4, which is the size of a single immunoglobulin domain (Ryu et al., 1990; Wang et al., 1990). Some antibodies, such VRC01-class antibodies, mimic CD4 binding with their heavy chains, allowing for their neutralization of most circulating HIV-1 isolates (Huang et al., 2016; Wu et al., 2010; Zhou et al., 2010); other antibodies, such as the glycan276-dependent antibody VRC40 (Cottrell et al., 2021), evolve favorable binding interactions with glycans surrounding the CD4bs. These antibodies, however, require unusual complementarity determining regions (CDRs) or extensive somatic hypermutation (SHM), and generally developed only after a long period of chronic infection, making them difficult to elicit by vaccination.

Some animals express specialized antibodies that appear to address the challenge of recessed binding surface. Cows, for example, have special long D genes that encode folded "knob" domains, which extend beyond the typical antibody surface (review in Stanfield et al., 2018). While immunization with mimics of the HIV-1 envelope (Env) trimer generates only autologous neutralization in rabbits, guinea pigs and non-human primate (Carrat and Flahault,

2007; Pauthner et al., 2019; Sanders et al., 2015; Wilson et al., 2000), immunization of cows elicits effective responses with substantial breadth, which arises from antibodies containing the knob domain (Sok et al., 2017). Members of the *Camelidae* family (camels, dromedaries, and llamas) produce – in addition to the standard double-chain immunoglobulins – a subclass of antibodies without light chains, called heavy chain-only antibodies (Hamers-Casterman et al., 1993). The single variable domain of these heavy-chain-only antibodies are termed VHHs, single domain antibodies, or nanobodies. A number of nanobodies that target the CD4bs of HIV-1 Env have been isolated from llamas immunized with soluble versions of the HIV-1 Env (Forsman et al., 2008; McCoy et al., 2012; McCoy et al., 2014; Strokappe et al., 2012; reviewed in Weiss and Verrips, 2019). These immunization-elicited nanobodies display a range of neutralization properties, from the extraordinary J3 with the ability to neutralize over 95% of circulating HIV-1 strains (McCoy et al., 2012) to A12, D7 and C8, with more limited neutralization breadths (Forsman et al., 2008; Hinz et al., 2010; Koh et al., 2010).

To gain insight into recognition by these nanobodies of the HIV-1 Env, we determined the cryo-electron microscopy (cryo-EM) structure of J3 in complex with HIV-1 Env trimer, along with crystal structures of nanobodies J3, A12, D7, and C8, in complex with HIV-1 gp120 core. We analyzed the recognition interface of these complexes and the structural features of nanobody binding to monomeric HIV-1 gp120 core versus trimeric HIV-1 Env ectodomain. Overall, the results provide molecular insight into the ability of llama nanobodies to recognize the recessed CD4bs and provide an explanation for the different neutralization profiles of the nanobodies, with conformational masking shielding the CD4bs – not only from two-chain human immunoglobulins – but also from most single-chain nanobodies.

Results

Cryo-EM structures of J3 in complex with the HIV-1 Env trimer stabilized in its prefusion-closed conformation reveals J3 mimicry of the CD4 receptor

We assessed neutralization of llama nanobodies, J3, A12, C8 and D7 (Forsman et al., 2008; McCoy et al., 2012) that target the CD4bs on a 208-strain panel of diverse HIV-1 viruses (Chuang et al., 2019). Assessment was carried out both in a monomeric nanobody format and in a bivalent IgG2a format (VHH linked to human Fc C_H2 and C_H3 domains with the llama IgG2a hinge region) (Figure S1A). Consistent with what has been reported (Forsman et al., 2008; McCoy et al., 2012), we observed the J3 nanobody to exhibit much broader and more potent neutralization than the other three nanobodies, with a breadth of 95% and a geometric mean IC_{50} of 0.256 µg/ml, better than VRC01 and soluble CD4 (Figure S1B and Table S1). In contrast to the broad and potent neutralization of J3, nanobodies A12, C8, and D7 only neutralized 37%, 24%, and 15%, respectively, of the 208-strain panel. In the IgG2a format, J3 displayed slightly higher neutralization breadth, while the IgG2a format of A12 showed lower neutralization breadth.

To provide the structural basis for the neutralization of HIV-1 strains by the llama nanobodies, we undertook structural analyses of the binding interactions between the nanobodies and Env trimer. We determined a cryo-EM structure of J3 in complex with BG505 DS-SOSIP Env trimer (clade A) at a nominal resolution of 3.6 Å (Figures 1 and S2; Table S2). The cryo-EM structure of the J3-Env trimer complex revealed J3 to bind in a recessed region of the trimer overlapping the CD4bs, with each J3 molecule interacting with two gp120 subunits with buried surface areas (BSAs) of ~941 $Å^2$ to one gp120 (primary site) and ~224 $Å^2$ to the other gp120 (secondary site) (Figure 1 and Table S3). Minor interactions with *N*-linked glycans at residues

N301 (~80 Å^2) and N197 (~55 Å^2) were also observed. J3 binding mimicked CD4 binding to the prefusion-closed Env trimer, both for the primary site of recognition as well as to the secondary site on the adjacent protomer (Liu et al., 2017): there was a shift of the J3 epitope toward the trimer apex (Figure 1B) and a shift of the adjacent protomer in the CD4 complex of the prefusion-closed Env trimer structure (PDB: 5U1F), although the same quaternary CD4bs residues were involved in binding J3. Overall, the J3 VHH occupied a position that overlapped spatially with that of CD4 D1 domain and of the heavy chain variable domain of VRC01 (Figure 1C); the smaller size of the VHH versus the double-headed immunoglobulin domain enabled it to bind the CD4bs with minimal interaction with surrounding glycan.

Cryo-EM structure of J3 with BG505 DS-SOSIP Env and crystal structure of J3 with gp120 core exhibit identical J3 orientations with strong interactions to CD4bs residues

To obtain higher resolution details of the J3-Env binding interactions, we determined a crystal structure of J3 in complex with HIV-1 gp120 extended core (core$_e$) (Kwon et al., 2012) of strain C1086 (clade C) at 2.55 Å resolution (Figure 2A, S3A and Table 1). The crystal structure of J3-gp120 core$_e$ complex superimposed well with the cryo-EM structure (Figures 2A and S3). J3 of both structures aligned well with a root-mean-square deviation (RMSD) of 0.79 Å for all aligned Cα atoms (residues 1 to 120). Most residues involved in binding, particularly CDR residues, had relatively low Cα deviations from the J3-Env trimer structure (Figure S3B). The gp120s of the structures had an RMSD of 2.8 Å for aligned Cα atoms of 335 residues. Most of the deviations were located near truncated regions in the gp120 core$_e$ construct, such as loops V1/V2 and V3, at residues 57-76 near trimer interface, or where either of the structures was disordered (Figures 2A, S3C, and S4A). All residues in gp120 involved in the primary site

binding interface of the cryo-EM structure were present in the gp120 $core_e$ construct, and most of them were located in regions with relatively low Cα deviations, except residues $H105_{gp120}$ and $I109_{gp120}$ in the middle of helix α1 (Figure S3C); we note however that helix α1 is proximal to the deleted V1V2 region of the gp120, and its C-terminal half shifted between the two structures (Figure S4A).

Despite the differences in the two structures, both revealed nearly identical binding interfaces at the primary binding site, involving the same set of residues from J3 and gp120 (Figures 2B,C and S5A). The interface BSAs for both structures were essentially identical, aside from the additional quaternary site observed from the cryo-EM structure (Table S3). Nonetheless, the crystal structure revealed more hydrogen bonds between CDRs and gp120 (Figure S5B,C). This was partly due to the higher resolution of the crystal structure and partly to the absence of obstruction from neighboring protomers allowing for more intimate interactions between J3 and gp120.

The binding of J3 involved all three CDRs at the primary binding site on the trimer surface as well as the N terminus and framework regions (FRs) 1 and 3 at the secondary binding site (Figure 2B). The primary-site binding interactions were dominated by the CDR3 (Figure 2C), which covered over 600 $Å^2$ of BSA (Table S3). CDR3 interacted with the N terminus of helix α5, loop β20-β21, CD4-binding loop, strands β23 and β24 flanking V5, and D-loop. CDR1 and CDR2 contributed 166 and 152 $Å^2$ of BSA, respectively. CDR1 interacted with the N terminus of helix α5 and the middle of α1, and CDR2 interacted with a few residues of β21 and the CD4-binding loop. Minor interactions were observed to glycan197 from CDR2 residue 57, FR3 residues 64 and 65 (~55 $Å^2$). There were seven or more potential hydrogen bonds, most of them from CDR3 and many involving backbone atoms. Notably, the side chain of $Y99_{J3}$ bound

in a hydrophobic pocket in gp120, mimicking CD4 residue F43, which makes critical hydrophobic interactions with gp120 (Kwong et al., 1998; Ryu et al., 1990; Wang et al., 1990). The equivalent position in VRC01 contains a glycine, although replacement of the glycine with phenylalanine, tyrosine, or tryptophan increases VRC01 potency (Diskin et al., 2011); the equivalent residue of VRC03 is a tryptophan (Wu et al., 2011), which mimics CD4 F43 binding in the hydrophobic pocket. Another notable interaction was a potential salt bridge between side chains of $H58_{J3}$ and $D368_{gp120}$. Earlier studies showed that D368R mutation in gp120 abolishes J3 binding (McCoy et al., 2012). The distance between the side chains was ~4.0 Å in the cryo-EM structure; however, this distance was at a normal hydrogen bond distance of 2.7 Å in the crystal structure of the J3-gp120 $core_e$ complex. This salt bridge mimicked the critical contact between residues R59 of CD4 and $D368_{gp120}$. Unlike the prefusion-closed stabilized trimer used in the cryo-EM study, Env trimer on viral surface is metastable, and thus this salt bridge likely plays a role in J3 neutralization activity.

The binding interactions at the secondary site on the neighboring protomer was less extensive, involving van der Waals interactions, one hydrogen bond between side chains of $Q3_{J3}$ and $E62_{gp120}$ at 3.5 Å, and a salt bridge from the N terminus of J3 to $E62_{gp120}$ at 3.7 Å in the cryo-EM structure (Figure 2D). One patch of the secondary site involved residues $E1_{J3}$, $Q3_{J3}$, $R25_{J3}$, and $I28_{J3}$ interacting with $E62_{gp120}$, $T63_{gp120}$, and $E64_{gp120}$; another patch of the interface involved residues $N73_{J3}$ and $A74_{J3}$ with $P206_{gp120}$ and $K207_{gp120}$. $F17_{J3}$, $L18_{J3}$, and $R19_{J3}$ interacted with a mannose residue of glycan301, which accounted for ~80 $Å^2$ of BSA.

To validate the J3-binding interactions observed in the crystal and cryo-EM structures, we prepared nine J3 mutants (Table S4), N30A and A31A at CDR1, H58A at CDR2, A74Y at FR3, and K96A, T98R, Y99G, N100CR, and N100EQ at CDR3, and analyzed their binding to

Env trimer by surface plasmon resonance (SPR) (Figure S5D). Mutations H58A, T98R, and Y99G knocked out binding, whereas other mutations reduced the affinity ~18-75 folds. These results confirmed the structural analysis above for critical residues involved in J3-Env binding interactions.

Overall, J3 had a size similar to that of the domain 1 of CD4, enabling it to bind in the same location as CD4 without steric hindrance from glycans surrounding the CD4bs. Binding of J3 recapitulated many of the critical CD4 interactions, including the many hydrogen bonds involving main chain and side chain atoms, tyrosine binding in the hydrophobic pocket, and the salt bridge to $D368_{gp120}$. These characteristics of J3 binding allowed the nanobody to exhibit remarkable breadth and potency.

Crystal structures of A12 in complex with gp120 $core_e$ reveal a binding site shifted to the interface between Env protomers

To elucidate the mechanism underlying the different neutralization profiles between the nanobodies, we determined crystal structures of A12 alone at 1.5 Å resolution and in complex with gp120 $core_e$ from strain C1086 (clade C) at 2.6 Å resolution (Figure 3 and Table 1). Similar to J3, A12 bound near the CD4bs with ~900 $Å^2$ of BSA, and the binding interface was dominated by CDR3 interactions (Figures 3 and Table S3). However, relative to the J3 epitope, the A12 epitope shifted away from the CD4-binding loop and D loop, with substantial interactions with helix α1 (Figure 3A); this shift would put the A12 epitope closer to the protomer interface of Env trimer. The gp120 component of the A12-gp120 $core_e$ complex was nearly identical to that of the J3-gp120 $core_e$ complex, with an identical orientation of helix α1, which deviated from that of the same helix in the Env trimer complex cryo-EM structure (Figure

S4B,C), presumably a consequence of the V1V2 truncation of the gp120 core construct. However, unlike the J3 complex crystal structure, the bridging sheet was disordered in the A12-gp120 complex (Figure S4B,C).

Disordering of the bridging sheet, especially the loop between strands β20 and β21, exposed the C terminus of helix α1, allowing this helix to interact extensively with A12. The N terminus (residue $A1_{A12}$) had charge-charge interactions with the side chain of $E102_{gp120}$ of helix α1, and residues from the N terminus and CDR1 interacted with residues along one side of entire helix α1 (Figure 3B). CDR3 interacted with the interface between the inner and outer domains of gp120 in a manner reminiscent of the heavy chain CDR3 of antibody F105 (Chen et al., 2009). The $L99_{A12}$ side chain bound in a hydrophobic pocket, similar to that of $Y99_{J3}$. Residue $R97_{A12}$ had charge-charge interactions with the side chain of $E370_{gp120}$ and likely with also $D368_{gp120}$, which formed a salt bridge in CD4 binding as well as in J3 binding as described above.

Crystal structures of C8 and D7 in complex with gp120 reveal a binding mode similar to that of A12

As described above, two other llama nanobodies, C8 and D7, have been found to target the CD4bs with cross-clade neutralization activities, but with somewhat limited breadth. To obtain a structural explanation of their neutralization activities, we determined crystal structures of C8 in complex with HxB2 (clade B) gp120 $core_e$ at 2.76 Å resolution and D7 in complex with RHPA (clade B) gp120 $core_e$ at 1.76 Å resolution (Figure 4 and Table 1). Both nanobodies bound near the CD4bs, but their epitopes shifted toward the inner domain relative to the J3 epitope, similar to that of A12 (Figure 4A). C8 bound at a higher position toward the apex, whereas D7 bound at a position identical to that of A12.

Binding of C8 ordered a segment of sequence of gp120, residues 422-430, which formed an extended structure and made extensive interactions with C8 (Figures 4B left panel and S4C). This segment of sequence was disordered in the A12 complex, and the corresponding sequence segment in the J3 and CD4 complexes formed the β20-loop-β21 hairpin, which played an important role in binding J3 and CD4. C8 also interacted with a segment of sequence downstream of helix α1, which was disordered in the A12 complex, but became ordered and formed a β hairpin upon binding of C8 (Figures 4B left panel and S4C). As a result of these extra binding surface, the C8 epitope was larger with close to 1300 $Å^2$ of BSA (Table S3), and it shifted toward the apex with ~50% overlap with the J3 epitope (Figure 4B, left panel).

The structure of the D7-gp120 $core_e$ complex revealed a binding mode similar to that of the A12 complex, with a total interface of ~980 $Å^2$, dominated by CDR3 with minor contributions from CDR1 and the N terminus (Figure 4B right panel and Table S3). Relative to the J3 epitope, the D7 epitope shifted away from the outer domain and focused mostly on the inner domain, in a manner similar to that of A12. The bridging sheet was disordered, and the stretch after helix α1 and before the truncated V1V2 loop was also mostly disordered, making helix α1 accessible to D7 (Figure S4D). The high-resolution structure allowed for precise definition of binding interactions (Figure 4B right panel). The N terminus ($Q3_{D7}$) interacted with the N-terminal first turn of helix α1, and CDR1 interacted with the rest of the exposed side of α1, making one salt bridge from $R27_{D7}$ to $D107_{gp120}$. CDR3 made extensive interactions with helices α1, α3, and α5, residues 254-257 in loop B (between β8 and β9), and the β-sheet of strands β16, β17, and β19, with salt bridges $K95_{D7}$ to $E368_{gp120}$ and $R97_{D7}$ to $E370_{gp120}$, and one hydrogen bond from the main-chain carbonyl of $L99_{D7}$ to the OH group of $Y384_{gp120}$. As in the A12 complexes, the $L99_{D7}$ side chain bound in a hydrophobic pocket, in a manner similar to that

of $Y99_{J3}$. Residue $R97_{D7}$ had also charge-charge interactions with the side chain of $D368_{gp120}$. Overall, D7 had extensive interactions with gp120 $core_e$ very similar to those observed in the A12-gp120 $core_e$ complexes.

Nanobodies D7 and A12 were 92% identical in sequence (Figure S6A). There were three different residues each in CDR1 and CDR3, likely resulting in stronger binding of D7 in the regions around helix α1 and weaker binding of D7 with α5 residues and the CD4-binding loop of gp120, compared with the binding of A12 (Figure S6). The D7 CDR3 shifted slightly toward helix α1 (Figure S6B). These differences likely resulted in D7 binding and neutralization to rely more strongly on the easily open conformation of the Env trimer (see details below), explaining its lower neutralization breadth relative to that of A12.

Binding mode of J3 is compatible with the prefusion-closed Env trimer conformation, with which A12, C8, and D7 appear to have substantial clashes

The above structural analyses indicated that all four nanobodies bound in the CD4bs with substantial epitope overlap and had comparable binding interface areas, except C8, which had a substantially larger interface (Table S3). Yet, their neutralization potency and breadth were different, with J3 being far better than the others. To understand the underlying mechanism for their difference in neutralization activity, we superimposed the crystal structures of the nanobodies in complex with gp120 $core_e$ on the cryo-EM structure of J3 in complex with the prefusion-closed-stabilized Env trimer (Figure 5).

As described above, J3 bound at the identical position in both crystal and cryo-EM structures. Modeling of the J3 of the crystal structure onto the cryo-EM structure by aligning the gp120 subunit revealed a footprint on Env trimer essentially identical to the epitope of the J3-

Env complex, including the interfaces on both adjacent protomers and the minor interactions with glycans at N301 and N197, without clashes (Figure 5A). In contrast, the other three nanobodies, A12, C8, and D7, when modeled on the Env trimer based on their binding modes with gp120 in the crystal structures, revealed substantial clashes (Figure 5, B-D). These three nanobodies would bind deeply in the interprotomer interface of the trimer and clashed into the adjacent protomer, where - by contrast - J3 had a favorable quaternary contact; C8 bound higher toward the trimer apex and had an additional clash site on the adjacent protomer between glycan197 and glycan301 (Figure 5C). The majority of the clashes, however, were located at the primary binding site near the inter-protomer interface and toward the apex. These clashing residues of the Env trimer were either absent or disordered in the gp120 $core_e$ complex structures of A12, C8, and D7 (Figure S4, B-E), enabling these nanobodies to bind to gp120 surface that were otherwise covered in the prefusion-closed conformation of the Env trimer. This binding mode would require the Env trimer to be in an open conformation with gp120 partially disordered for the nanobodies to bind.

Different binding modes of the nanobodies correlate with their neutralization activities

To determine if the binding modes of the nanobodies in the gp120 $core_e$ complexes were related to their neutralization activities, we analyzed their neutralization fingerprints (Georgiev et al., 2013). We observed A12, C8 and D7 to cluster with antibodies 447-52D, 48D, 17b, and F105, as a subgroup of antibodies that preferentially neutralized strains with Tier 1-neutralization resistance (Posner et al., 1993; Thali et al., 1993; Zolla-Pazner et al., 2004) (Figure 6A). This cluster was next to a subgroup of sCD4, CD4-Ig, and another Tier 1-neutralizing antibody 3074 (Agarwal et al., 2011). On the other hand, the J3 neutralization fingerprint was distantly related

to the entire cluster of CD4, Tier 1-neutralizing antibodies, and nanobodies A12, C8, and D7. Despite its binding mode with prefusion-closed Env being highly similar to J3, CD4 binding induces the open conformation of Env (Kwong et al., 1998; Ozorowski et al., 2017). As indicated in above structural analyses, the epitopes of A12, C8, and D7 included surfaces that would be buried in the prefusion closed Env, indicating their binding to induce more open conformations of Env. This similar binding feature between nanobodies A12, C8, and D7 and the CD4 receptor likely explained their neutralization fingerprints being closely clustered. Notably, soluble CD4 has a much higher neutralization breadth than those of A12, C8, D7, and Tier 1-neutralizing antibodies, yet showed a similar pattern of preference for the viral strains (Figures 6 and S1, Table S1), indicating similar modes of interaction with Env trimer.

A subset of viruses was neutralized strongly by A12, C8, and D7, with almost all viruses neutralized by D7 being also neutralized by A12 and C8 (Figures 6B, S1 and S7). These viral strains were mostly neutralization sensitive strains, designated on the basis of their sensitivity to the Tier 1-neutralizing antibodies 17b, 48d, F105, 447-52D, and 3074. A resistant strain is designated as having IC_{50} >50 μg/ml for antibodies 17b, 48d, F105, and 447-52D; and IC_{80} >50 μg/ml for antibody 3074 (Cheng et al., 2019). C8 in IgG2a bivalent format also strongly neutralized a few strains that were weakly or not neutralized by A12 or D7; some of these strains were also preferentially neutralized by A12 in IgG2a format (Figure 6B). However, the IgG2a format substantially decreased neutralization for A12 to 15% breadth (although it improved slightly the neutralization breadth for J3 Figure S1 and Table S1). J3 had a different neutralization profile and neutralized both sensitive and resistant strains (Figures 6B and S1).

Overall, the neutralization properties of these nanobodies were consistent with their modes of recognition, although other factors, such as affinity maturation as demonstrated by the

difference between A12 and D7, can also affect neutralization capacity. The binding mode of the nanobodies A12, C8, and D7 in the gp120 complex indicated that these nanobodies could only bind and neutralize HIV-1 strains that had flexible Env trimers, which could adopt more open conformations and thus allow these nanobodies to bind in a fashion similar to their binding to the gp120 core in the crystal structures. On the other hand, J3 mimicked CD4 binding to the prefusion-closed Env trimer and was able to neutralize the majority of HIV-1 circulating strains.

Discussion

Nanobodies have been identified to neutralize effectively diverse strains of influenza A virus (Laursen et al., 2018), beta-coronaviruses like SARS-CoV-2 (Koenig et al., 2021; Wrapp et al., 2020; Xu et al., 2021), and HIV-1 (McCoy et al., 2012). While the structural basis for the broad neutralization of influenza A and SARS-CoV-2 has been determined, this was lacking for HIV-1. Here we provide the structural basis for the near-pan neutralization of HIV-1 by the J3 nanobody, as well as the more limited neutralization of by A12, C8, and D7 nanobodies.

J3 was isolated from a llama immunized with gp140 Env trimer (McCoy et al., 2012), and its binding to Env trimer mimicked that of the CD4 receptor with the prefusion-closed conformation of Env. On the other hand, A12, C8, and D7 derived from a llama immunized with recombinant gp120 (Forsman et al., 2008). Recombinant gp120 expresses as a monomer and folds into a conformation that differs from gp120 in the prefusion closed Env trimer, resulting in the exposure of select surfaces, such as the C-terminal half of helix α1, which are mostly inaccessible on the viral particles. These exposed surfaces appear to be highly immunogenic and elicit antibodies that are non-neutralizing or have limited neutralizing capacity. Our results show that even the smaller nanobodies tend to recognize the target CD4bs in the monomeric gp120

context in ways that require co-recognition of neighboring immunogenic surfaces, whose conformations differ in the prefusion-closed conformation. Conformation masking (Kwong et al., 2002) can thus prevent the binding of most nanobodies, with precise mimicry of CD4 by J3 appearing to be one of the few ways to effectively recognize the CD4bs The differences in the Env-binding modes and neutralization profiles of the llama nanobodies analyzed in this study highlight the importance of designing immunogens with optimal structure and conformation, to present viral antigens in an appropriate conformational context as a first step in eliciting antibodies of desired potency and protective efficacy.

For heavy-chain-only antibodies, the single variable domain (nanobody) is solely responsible for antigen recognition. Nanobodies have the potential advantage of being smaller in size relative to the antigen-binding fragment (Fab) of standard two-chain immunoglobulins, allowing them to access smaller recessed binding surfaces of viral pathogens, which often evolve features that avoid antibody-mediated recognition. As described above, J3 from immunization of llama, being similar in size to the CD4 N-terminal domain, was able to mimic CD4 binding and neutralize potently over 95% of circulating HIV-1 strains, with relatively low affinity maturation. While two-chain antibodies such as those of the VRC01 class can be developed to with even more broad and potent neutralization of HIV-1 (Kwon et al., 2021; Zhou et al., 2015), their developmental pathways often require low frequency events, such as extensive SHM or unusual recombination. Thus, while human antibodies can achieve broad neutralization of HIV, the smaller size of nanobodies can be advantageous over the larger Fabs in recognizing receptor-binding sites. It will be interesting to see if – based on the structural finding presented here – even more potent and broadly reactive nanobodies can be identified against HIV-1, either through immunization of llamas or nanomice or screening of nanobody libraries with prefusion-

closed Env trimers of divergent strains. An additional advantage of nanobodies is their ease in adapting to use as multivalent neutralizing reagents, and it will be interesting to see if nanobodies such as J3 can be used in combination with other broad and potent HIV-1 neutralizing antibodies in prevention or treatment of HIV-1.

Consortia

The VRC Production Program includes Nadia Amharref, Nathan Barefoot, Christopher Barry, Elizabeth Carey, Ria Caringal, Kevin Carlton, Naga Chalamalsetty, Adam Charlton, Rajoshi Chaudhuri, Mingzhong Chen, Peifeng Chen, Nicole Cibelli, Jonathan W. Cooper, Hussain Dahodwala, Marianna Fleischman, Julia C. Frederick, Haley Fuller, Jason Gall, Isaac Godfroy, Daniel Gowetski, Krishana Gulla, Vera Ivleva, Lisa Kueltzo, Q. Paula Lei, Yile Li, Venkata Mangalampalli, Sarah O'Connell, Aakash Patel, Erwin Rosales-Zavala, Elizabeth Scheideman, Nicole A. Schneck, Zachary Schneiderman, Andrew Shaddeau, William Shadrick, Alison Vinitsky, Sara Witter, Yanhong Yang, and Yaqiu Zhang.

Acknowledgments

We thank J.R. Mascola for advice and support on antibody neutralization assays, J. Stuckey for assistance with figures, members of the Electron Microscopy Group at the New York Structural Biology Center for assistance with data collection, and members of the Virology Laboratory, Vaccine Research Center, for discussions and comments on the manuscript. Support for this work was provided by the Intramural Research Program of the Vaccine Research Center, National Institute of Allergy and Infectious Diseases. We thank J. Baalwa, D. Ellenberger, F. Gao, B. Hahn, K. Hong, J. Kim, F. McCutchan, D. Montefiori, L. Morris, E. Sanders-Buell, G.

Shaw, R. Swanstrom, M. Thomson, S. Tovanabutra, C. Williamson, and L. Zhang for contributing the HIV-1 envelope plasmids used in our neutralization panel, and N. Jean-Baptiste, R. Carroll, B. Flach, K. McKee, C. Moore, G. Padilla, J. Rathmann, S. O'Connell, S. O'Dell, S.D. Schmidt, C. Whittaker, and A.B. McDermott for assistance with neutralization assessments on the 208-strain panel. Use of sector 22 (Southeast Region Collaborative Access team) at the Advanced Photon Source was supported by the US Department of Energy, Basic Energy Sciences, Office of Science, under contract number W-31-109-Eng-38. Some of this work was performed at the Simons Electron Microscopy Center (SEMC) and the National Resource for Automated Molecular Microscopy, located at the New York Structural Biology Center, supported by grants from the Simons Foundation (SF349247), NYSTAR, and the NIH National Institute of General Medical Sciences (GM103310).

Author Contributions

T.Z. refined and analyzed crystal structures; L.C. grew crystals, collected diffraction data, and partially refined crystal structures; J.G. determined the cryo-EM structure; S.W. analyzed structural and neutralization data and co-wrote the manuscript; Y.D.K. provided gp120 core used in crystallization; B.C.L., M.K.L., and N.D.R. provided neutralization data; R.R. performed neutralization fingerprint analysis; E.D.S. prepared J3 mutants; Y.Y. and B.Z. produced the nanobody proteins; A.F., L.E.M., L.R., C.T.V., and R.A.W. provided nanobody sequences; V.P.P. provided Env trimer for cryo-EM; P.D.K. oversaw the project, and with L.S. and S.W. co-wrote the manuscript, with all authors providing revisions and comments.

Declaration of Competing Interests

The authors declare no competing interest.

Figure titles and legends

Figure 1. Cryo-EM structure of J3 in complex with a Clade A-stabilized Env trimer reveals J3 to mimic CD4 binding to two adjacent protomers on the prefusion-closed trimer.

(A) Overall structure of the J3-BG505 DS-SOSIP complex. J3 is shown in cartoon representation colored green, and BG505 DS-SOSIP is shown as surface in shades of gray except for the J3-binding epitope, which is highlighted in forest-green. Glycans are shown in sticks.

(B) J3 epitope highlighted in forest-green on the surface of Env trimer, in the same orientation as in (A) left panel. The epitope was defined as Env atoms within 5.5 Å of J3. Yellow outline indicates the footprint of CD4 mapped on the trimer surface of the J3 complex, by aligning the gp120 domain of the CD4-gp120 complex with that of the J3 complex. J3 binds across two adjacent protomers, slightly higher towards the trimer apex than CD4bs, but with substantial overlap and similar total contact surface area.

(C) Superposition of the J3 complex with CD4 (PDB: 2NY1) and VRC01 (PDB: 5FYJ) complexes, by aligning the gp120 domain. Only the J3 complex, CD4 D1 domain, and VRC01 variable domains are shown for clarity. VRC01 is shown in marine blue for the heavy chain and slate blue for the light chain. CD4 is shown in yellow.

See also Figure S2 and Table S2.

Figure 2. Cryo-EM structure of J3 with BG505 DS-SOSIP Env and crystal structure of J3 with clade C gp120 core exhibit identical J3 orientation with strong binding interactions to CD4bs residues.

(A) Alignment of the crystal structure of J3-C1086 gp120 core with the cryo-EM structure of J3-BG505 DS-SOSIP complex. The structural alignment was based on the Cα atoms of both gp120 and J3 residues. The cryo-EM structure is shown in green for J3 and gray for Env trimer. The gp120 core complex crystal structure is shown as orange round tubes with the diameter representing the pairwise Cα distances between the two structures. Cα distances greater than 10 Å and those unaligned residues are plotted as 10-Å diameter tubes to avoid obscuring the deviations of the aligned residues. The nanobodies aligned well; most of the gp120 core also aligned well, except residues 57-76 near trimer interface, residues flanking the deleted loops V1/V2 and V3 in the gp120 extended core construct, and residues at where either structure was disordered.

(B) J3 paratope observed in the J3-BG505 DS-SOSIP Env complex. The paratope involved three CDRs to interact with the primary binding site and FR1, FR3, and N terminus with a secondary site on an adjacent protomer. There were some minor binding interactions between residues in FR3 and glycan197 in the primary site, and the secondary site binding involved glycan301. Nanobody residues involved in binding are highlighted in magenta.

(C) Detailed binding interactions in the primary site. BG505 Env is shown as ribbon and transparent surface, with the epitope surface and atoms involved in binding highlighted in forest green. Nanobody side chains involved in binding are shown in sticks, with atoms within 5.5 Å of Env highlighted in magenta. Main chain carbonyl involved in hydrogen

bonds are shown in sticks. Hydrogen bonds are shown as yellow dashed lines. $H58_{J3}$ had a potential charge-charge interaction with $D368_{gp120}$, shown as an orange dashed line, with a distance of ~4.0 Å. $Y99_{J3}$ side chain bound in a hydrophobic cavity, with its OH group having a hydrogen bond to the side chain of $D370_{gp120}$ at the bottom of the cavity.

(D) Detailed binding interactions in the secondary site at the adjacent protomer. Quaternary interactions involved FR1, FR2, and N terminus of J3. Residues involved in the quaternary site binding are labeled.

See also Figures S3-S5, and Tables S2-S4.

Figure 3. Crystal structure of A12 in complex with gp120 core reveals a binding site shifting toward the interface between protomers relative to the J3 epitope.

(A) Overall crystal structure of A12 in complex with gp120 $core_e$ from clade C in comparison with that of J3. HIV-1 gp120 is depicted in light gray semi-transparent surface with the nanobody epitopes colored forest green; nanobodies are depicted as ribbon diagrams with A12 colored marine-blue and J3 colored orange. The gp120 structures of both complexes are shown at the same viewing orientation, also the same orientation as the light-gray-colored gp120 subunit in Figure 1A left panel for easy comparison. The A12 binding site shifted away from the outer domain relative to that of J3.

(B) Zoom-in view showing the detailed nanobody-gp120 interactions. The structures are shown in the same location and viewing angle, at a 45° rotation along the vertical axis relative to that in (A). Nanobody side chains involved in binding are shown in sticks, with atoms within 5.5 Å of Env highlighted in magenta. Similar to J3, the primary interactions for A12 were dominated by CDR3, with CDR1 providing additional

contacts. The contact surface on gp120 for A12 shifted away from the outer domain. The bridging sheet was disordered in the A12 complexes, allowing the nanobody to bind to a portion of gp120 surface that was otherwise buried by the bridging sheet and part of gp120 absent in the gp120 $core_e$ construct, or blocked by the neighboring protomer in the Env trimer.

See also Figure S3 and Table S3.

Figure 4. Crystal structures of C8 and D7 in complex with gp120 $core_e$ reveal a binding mode similar to that of A12, with a binding site shifting toward the interface between Env protomers.

(A) Overall crystal structures of C8 and D7 in complex with gp120 $core_e$ from clade B HxB2 and RHPA, respectively. In the two left panels, the gp120 structures are depicted as semi-transparent surface with the nanobody epitopes highlighted in forest-green; nanobodies are depicted as ribbon diagrams with C8 in slate-blue and D7 in cyan. The rightmost panel shows superposition of the two structures in ribbon diagram; gp120 is in dark gray for the C8 complex and light gray for the D7 complex. The structures are shown in the same viewing orientation as in Figure 3A for easy comparison. C8 and D7 bound at a site similar to that of A12, and shifted away from the outer domain relative to the J3 epitope. C8 interacted with some structural elements near apex that were disordered in the A12- and D7-gp120 complexes, and it bound at a slightly higher position than A12 and D7.

(B) Zoom-in view at the epitope for the nanobody-gp120 detailed interactions. The structures are shown in the equivalent regions, viewed at a 45° rotation along the vertical axis relative to that in (A). The C8 and D7 epitopes are colored forest-green, with the footprint

of J3 shown as green outline for comparison. Nanobody side chains involved in binding are shown in sticks, with atoms within 5.5 Å of Env highlighted in magenta. Hydrogen bonds are shown as yellow dashed lines. Similar to A12, the binding interactions for D7 were dominated by CDR3, with CDR1 providing additional contacts. However, C8 has a different set of paratope residues comprising CDR1, CDR2, CDR3, and FR3. The contact surface on gp120 for both nanobodies shifted away from the outer domain, and part of their contact surface on gp120 would be otherwise covered by the bridging sheet disordered in the crystal structures and by part of gp120 not present in the gp120 extended core construct, or blocked by the neighboring protomer in the Env trimer.

See also Figure S3 and Table S3.

Figure 5. The binding mode of J3 in the gp120 core complex is compatible with binding to the prefusion-closed Env trimer, whereas A12, C8, and D7 have substantial clashes with Env trimer.

The nanobodies in crystal structures of gp120 $core_e$ complex, (A) J3-C1086, (B) A12-C1086, (C) C8-HxB2, and (D) D7-RHPA, were modeled onto the cryo-EM structure of J3-BG505 DS-SOSIP Env trimer by superposition of the gp120 domain using PyMOL. Env trimer surface around the binding site is shown with the gp120 of the primary binding site colored light gray and the adjacent protomer colored darker gray. The nanobody footprint on the trimer surface was defined as atoms within 5.5 Å of the nanobody and colored light teal; the trimer surface clashing with the modeled nanobody was defined as atoms within 2.0 Å of the nanobody and colored red. The J3 epitope as defined in the cryo-EM structure is shown as green contour for comparison.

Figure 6. Nanobodies A12, C8 and D7 neutralize mostly neutralization sensitive viruses, whereas J3 exhibits broad neutralization against both tier 1-neutralization sensitive and tier-2 neutralization resistant strains.

(A) Neutralization fingerprint analysis. (B) Neutralization IC_{50} of viruses neutralized by A12, C8 and D7. Nearly all viruses neutralized by D7 were also neutralized by A12 and C8. J3 had a different pattern of neutralization profile, and some viruses strongly neutralized by J3 were not neutralized by A12, C8, or D7. For comparison, neutralization data are also shown for soluble CD4 and human antibodies that preferentially recognize the open conformation of Env such as the CD4bs antibody F105, V3-antibodies 3074 and 447-52D, and CD4-induced antibodies 17b and 48d.

See also Figures S1 and S7, and Table S1.

Table 1. Crystallographic data and refinement statistics.

	A12-C1086	A12	J3-C1086	C8-HxB2	D7-RHPA
PDB ID	7RI2	3R0M	7RI1	7R74	7R73
Data collection					
Space group	$P4_32_12$	$P2_12_12_1$	$P4_22_12$	C2	C2
Cell constants					
a, b, c (Å)	66.6, 66.6, 266.9	57.9, 64.3, 81.5	119.1, 119.1, 110.5	121.1, 87.4, 91.6	130.5, 62.0, 64.0
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 109.1, 90	90, 108.2, 90
Resolution (Å)	50-2.80	50-1.50	50-2.55	50-2.75	31.4-1.76
	(2.90-2.80)*	(1.55-1.50)	(2.64-2.55)	(2.80-2.75)	(1.82-1.76)
R_{merge}	12.4 (34.1)	11.6 (47.0)	10.2 (52.8)	13.9 (36.8)	8.6 (46.4)
$I / \sigma I$	21.1 (3.4)	17.8 (2.2)	22.5 (1.6)	8.6 (1.8)	14.2 (1.8)
Completeness (%)	85.2 (47.6)	95.5 (74.9)	99.1 (91.5)	94.0 (50.2)	92.7 (53.2)
Redundancy	11.5 (7.6)	6.3 (3.7)	6.6 (7.2)	3.2 (1.7)	3.3. (1.8)
Refinement					
Resolution (Å)	23.5-2.8	34.4-1.5	46.2-2.55	49.6-2.76	31.4-1.76
	(2.9-2.8)	(1.55-1.50)	(2.64-2.55)	(2.86-2.76)	(1.82-1.76)
No. reflections	13,351 (717)	46,933 (3616)	25,795 (2369)	21,711 (1251)	44,447 (2592)
R_{work} / R_{free} (%)	27.6/29.0	19.1/21.8	17.5/22.4	21.3/27.1	18.2/21.9
	(40.0/43.2)	(28.3/32.2)	(21.0/26.4)	(29.7/33.0)	(25.6/31.6)
No. atoms					
Protein	3278	1952	3657	6426	3254
Ligand/ion	112	30	123	252	207
Water	0	353	134	81	143
B-factors					
Protein	150.7	22.4	50.8	50.6	54.9
Ligand/ion	173.9	35.6	60.8	71.7	76.1
Water	N/A	36.9	43.4	43.7	51.1
R.m.s. deviations					
Bond lengths (Å)	0.003	0.005	0.008	0.003	0.013
Bond angles (°)	0.680	0.948	0.950	0.57	1.24
Ramachandran					
Favored regions (%)	96.0	99.6	95.9	94.0	97.0
Disallowed regions (%)	0.5	0	0	0.5	0.5

* Values in parentheses are for the highest-resolution shell.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Peter D. Kwong (pdkwong@nih.gov).

Materials availability

Plasmids generated in this study are available upon request.

Data and code availability

Cryo-EM maps have been deposited to the EMDB with accession codes EMD-23480 and fitted coordinates have been deposited to PDB with accession codes 7LPN. Crystal structures have been deposited to PDB with accession codes 7RI1, 7RI2, 7R73, 7R74 and 3R0M. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines

HEK293F, 293T, and FreeStyle 293-F cells were purchased from Thermo Fisher Scientific. The cells were used directly from the commercial sources following manufacturer suggestions as described in detail below.

METHOD DETAILS

Preparation of llama nanobodies

The codon-optimized genes of the nanobodies were synthesized and cloned into the XbaI/BamHI sites of pVRC8400 expression vector containing a N-terminal llama leader sequence (ATGGAGCTGGGGCTGAGCTTGGTGGTCCTGGCTGCTCTTTTACAGG GTGTCCAGGCT) and a C-terminal His tag (GeneArt, Regensburg, Germany). Proteins were produced by transient transfection using 293fectin (Invitrogen, Carlsbad, CA) in 293F cells (Invitrogen) maintained in serum-free free-style medium (Invitrogen). Culture supernatants were harvested 4 - 5 days after transfection, filtered through a 0.45 μm filter, and concentrated and buffer-exchanged into 350 mM NaCl, 20 mM Tris (pH 7.5). Proteins were purified by Co-NTA (cobalt-nitrilotriacetic acid) chromatography method using a HiTrap IMAC HP column (GE Healthcare, Piscataway, NJ), followed by a Hiload 26/60 Superdex S200 prep grade size exclusion column. For IgG2A format nanobodies, a hinge sequence, EPKIPQPQPKPQPQPQPQPKPQPKPEPECTCPKCP, was added next to the VHH and followed by human gamma 2a Fc domains C_H2 and C_H3. The J3 paratope mutations were prepared by GeneImmune Biotechnology LLC (Rockville, MD), using the primers listed in Table S4 and pVRC8400-J3 plasmid as template. The mutant nanobodies were expressed and purified as described above.

Preparation of HIV-1 gp120s and Env trimer

The codon-optimized genes encoding residues 44-492 of gp120 with specific deletions at the V1/V2 and V3 regions (Kwong et al., 1998) of clade B HxB2 and clade C1086 were synthesized with a leader sequence encoding mouse IL-2 MYSMQLASCVTLTLVLLVN. The genes were cloned into the XbaI/BamHI sites of the mammalian expression vector pVRC8400, and transiently transfected by using 293fectin (Invitrogen, Carlsbad, CA) in HEK 293 GnTi$^-$

(Invitrogen) maintained in serum-free free-style medium (Invitrogen). Culture supernatants were harvested 4 - 5 days after transfection, filtered through a 0.45 μm filter, passed through a 17b affinity column, eluted with IgG elution buffer (Pierce), and immediately neutralized by adding 1M Tris-HCl pH 8.5. The proteins were purified by a Hiload 26/60 Superdex S200 prep grade size exclusion column. The gp120 protein fractions were concentrated to 4-8 mg/ml, flash frozen in liquid nitrogen, and stored at -80 ºC until further use. The BG505 DS-SOSIP Env trimer for the cryo-EM structure determination of the J3-Env complex was produced using stable CHO cell lines and purified by non-affinity chromatography (Gulla et al., 2021).

Crystallization of gp120-VHH complexes

The gp120 cores from clade B HxB2 and RHPA and clade C C1086 were used to form complexes with VHHs for crystallization trials. The gp120s were deglycosylated with Endo H and further purified with a Concanavalin A (Sigma) column to remove glycosylated gp120. The gp120-VHH complexes were formed by mixing deglycosylated gp120 and VHHs (1:1.2 molar ratio) at room temperature for 30 minutes and purified by size exclusion chromatography (Hiload 26/60 Superdex S200 prep grade, GE Healthcare) with buffer containing 0.35 M NaCl, 2.5 mM Tris (pH 7.0), 0.02% NaN_3. Fractions with gp120-VHH complexes were concentrated to ~10 mg/ml, and used for crystallization screening. Three commercially available screens, Hampton Crystal Screen (Hampton Research), Precipitant Synergy Screen (Emerald BioSystems) and Wizard Screen (Emerald BioSystems) were used for initial crystallization trials of the gp120-VHH complexes. Vapor-diffusion sitting drops were set up robotically by mixing 0.1 μl of protein with 0.1 μl of precipitant solutions (Honeybee, DigiLab). Droplets were allowed to

equilibrate at 20 ºC and imaged at scheduled times with RockImager (Formulatrix). Robotic crystal hits were optimized manually using the hanging drop vapor-diffusion method.

Diffraction quality crystals were grown in droplets containing 1µl of protein mixed with 1 µl of reservoir solutions. A12-C1086 crystals were obtained in 24.2% (w/v) of PEG 8000 and 50 mM of KH_2PO_4, pH 7.8. Crystals of A12 were obtained in 0.1 M imidazole pH 6.5, 1.4 M ammonium sulfate and 12% iso-propanol. C1086-J3 crystals were obtained in 1.0 M Li_2SO_4 and 100 mM HEPES pH 7.5. RHPA-D7 crystals were obtained in 0.1 M Tris HCl pH 8.5 and 21% of PEG8000.

Determination of crystal structures

Single crystals were flash-frozen under a liquid nitrogen stream in cryo-protectant solutions. The best cryo-protection solution for the A12 VHH crystal contained 30% glycerol, 1.8 M of ammonium sulfate, and 100 mM of imidazole, pH 6.5. The cryo-protection solution contained 15% 2R, 3R-butanediol, 32 % (w/v) of PEG 8000 and 50 mM of KH_2PO_4, pH 7.8 was used for A12-C1086 complex. 15% of 2R, 3R-butanediol was mixed with 1.4 M Li_2SO_4 and 100 mM HEPES pH 7.5 for data collection of complex C1086-J3. Data sets at 1.8 Å, 2.76 Å, 2.8 Å and 2.55 Å resolution, respectively, for D7-RHPA, C8-HxB2, A12-C1086 and J3-C1086 complexes were collected at the Advanced Photon Source (beamline SER-CAT ID22 and BM22). The diffraction data were indexed, integrated, and scaled with the HKL2000 package (Otwinowski and Minor, 1997). The structures were solved by molecular replacement with Phaser (McCoy et al., 2007) in the CCP4 Program Suite (Collaborative Computational Project, 1994). Further refinement was carried out with PHENIX (Adams et al., 2010), starting with torsion-angle simulated annealing with slow cooling. Iterative manual model building was carried out with

COOT (Emsley and Cowtan, 2004) with maps generated from combinations of standard positional, individual B-factor and TLS refinement algorithms. X-ray data and refinement statistics are summarized in Table 1.

Cryo-EM and structural analysis

The J3 nanobody was incubated in molar excess with BG505 DS-SOSIP Env trimer at a final concentration of 2 mg/ml in PBS and the complex was deposited on a C-flat 1.2/1.3 carbon grid (protochip.com). The grid was vitrified using an FEI Vitrobot Mark IV with a wait time of 30 seconds, blot time of 3 seconds, blot force of 1 and humidity of 100%. Leginon (Suloway et al., 2005) software was used for data collection on a Titan Krios microscope equipped with a Gatan K2 direct detection device. Data were collected in movie mode with 10-second exposures and a total dose of 77.72 e–/Å^2. Data was pre-processed with Appion (Lander et al., 2009; Voss et al., 2010); frames were aligned and dose-weighted with MotionCor2 (Zheng et al., 2017). The CTF was estimated with CTFFind4 (Rohou and Grigorieff, 2015; Zhang, 2016), particles were picked using DoG Picker (Lander et al., 2009; Voss et al., 2010), and RELION (Scheres, 2012) was used for particle extraction. The particle stack was imported to CryoSPARC 2.15 (Punjani et al., 2017) for 2D classifications, ab initio 3D reconstruction, and nonuniform 3D refinement. The initial reconstruction was achieved using C1 symmetry; after confirming three nanobodies bound per Env trimer, C3 symmetry was then applied for the final non-uniform refinement. Model building through Coot was followed by simulated annealing and real space refinement in Phenix 1.18 (Adams et al., 2004) and iteratively improved with manual fitting of the coordinates in Coot (Emsley and Cowtan, 2004). Geometry and map fitting evaluation were assessed through

Molprobity (Davis et al., 2004) and EMRinger (Barad et al., 2015). PyMOL (www.pymol.org) and ChimeraX (Pettersen et al., 2021) were used to generate figures.

Env-pseudotyped virus neutralization assay

Antibody neutralization activity was measured by using HIV-1 Env-pseudoviruses to infect TZM-bl cells as described previously, on a panel of 208 HIV-1 Env-pseudoviruses using the single-round infection assay of TZM-bl cells (Chuang et al., 2019; Sarzotti-Kelsoe et al., 2014). Antibodies were serially diluted and incubated with pseudoviruses at 37 °C for 1h. The mixtures were then incubated with TZM-bl cells (0.5 million/ml) at 37 °C for 2 days. Infection levels were determined with Bright-Glo luciferase assay (Promega, Madison, WI), and the data were fitted with a 5-parameter nonlinear regression model to obtain IC_{50} and IC_{80} values, the antibody concentration required to inhibit infection by 50% and 80%, respectively. Sham medium in place of antibody was used as a control.

Surface Plasmon Resonance (SPR)

Binding affinities of J3 and its mutants to HIV-1 Env were assessed by SPR on a Biacore S-200 (GE Healthcare) at 25°C in HBS-EP+ buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.05% surfactant P-20), as described previously (Kong et al., 2019). Briefly, BG505 DS-SOSIP at 15 μg/ mL was captured in the sample channel at ~500 response units (RU) on a CM5 chip through binding to a mouse BG505-base-targeting antibody that was immobilized on the chip by amine coupling to a high density that saturates the chip surface. Serial three-fold diluted nanobodies were passed through the sample and reference channels for 180 s followed by a 300 s dissociation phase at 30 μl/min. The chip surface was regenerated by flowing 3M $MgCl_2$

solution for 30 s at a flow rate of 50 µl/min. Blank sensorgrams were obtained by injection of the same volume of HBS-EP+ buffer in place of the nanobody solutions. Sensorgrams of the concentration series were corrected with corresponding blank curves and fitted globally with Biacore S200 evaluation software using a 1:1 Langmuir model of binding.

Neutralization fingerprinting analysis

Antibody neutralization fingerprints were analyzed based on neutralization data of 208 HIV-1 viral strains, as described previously (Georgiev et al., 2013). Briefly, neutralization data of the antibodies were transformed into an antibody-antibody neutralization-correlation matrix by calculating the correlation coefficients between the ranked vectors of neutralization potency against each viral strain for all pairs of antibodies, using the Spearman rank correlation coefficient. The correlation vectors were clustered hierarchically to generate an antibody clustering tree. The clustering tree was input into Dendroscope (Huson and Scornavacca, 2012) to generate a neutralization fingerprint dendrogram. Selected antibodies of each category, such as site of vulnerability and antibody class, were included to show proper clustering of the categories in the neutralization fingerprint dendrogram.

QUANTIFICATION AND STATISTICAL ANALYSIS

Cryo-EM data were processed and analyzed using CryoSPARC. Cryo-EM structural statistics were analyzed with Phenix and Molprobity. Statistical details of experiments are described in Method Details or figure legends.

Supplemental Excel Table Title

Table S1. Neutralization IC_{50} and IC_{80} data of nanobodies J3, A12, C8, and D7 in IgG2a and single-domain VHH formats on the 208-strain panel, related to Figure 6.

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