Bacterially expressed HIV-1 gp120 outer-domain fragment immunogens with improved stability and affinity for CD4-binding site neutralizing antibodies

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Protein minimization is an attractive approach for designing vaccines against rapidly evolving pathogens such as human immunodeficiency virus, type 1 (HIV-1), because it can help in focusing the immune response toward conserved conformational epitopes present on complex targets. The outer domain (OD) of HIV-1 gp120 contains epitopes for a large number of neutralizing antibodies and therefore is a primary target for structure-based vaccine design. We have previously designed a bacterially expressed outer-domain immunogen (ODEC) that bound CD4-binding site (CD4bs) ligands with 3–12 μM affinity and elicited a modest neutralizing antibody response in rabbits. In this study, we have optimized ODEC using consensus sequence design, cyclic permutation, and structure-guided mutations to generate a number of variants with improved yields, biophysical properties, stabilities, and affinities ($K_D$) for various CD4bs targeting broadly neutralizing antibodies, including the germline-reverted version of the broadly neutralizing antibody VRC01. In contrast to ODEC, the optimized immunogens elicited high anti-gp120 titers in rabbits as early as 6 weeks post-immunization, before any gp120 boost was given. Following two gp120 boosts, sera collected at week 22 showed cross-clade neutralization of tier 1 HIV-1 viruses. Using a number of different prime/boost combinations, we have identified a cyclically permuted OD fragment as the best priming immunogen, and a trimeric, cyclically permuted gp120 as the most suitable boosting molecule among the tested immunogens. This study also provides insights into some of the biophysical correlates of improved immunogenicity.

Designing an effective vaccine against human immunodeficiency virus (HIV-1) is one of the most challenging scientific problems of this century. Elicitation of broadly neutralizing antibodies (bNAb$s$) is a desirable trait for any anti-HIV-1 vaccine. The HIV-1 surface envelope (Env) gp120 is the major target for vaccine design, because it contains sites for cellular receptor and co-receptor binding, and it is the primary target of the humoral immune response. The primary reason for the difficulty in generating an effective vaccine against HIV-1 lies in its extensive sequence variability. Moreover, the conserved epitopes targeted by broadly neutralizing antibodies are often discontinuous in sequence and heavily glycosylated. Approaches to focus the immune response toward specific conformational epitopes targeted by known bNAb$s$ against HIV-1 are desirable. Protein-minimization is an attractive approach for designing vaccines against rapidly evolving pathogens because it can help in focusing the immune response toward conserved, conformational epitopes present on complex protein targets. The outer domain (OD) of HIV-1 gp120 contains epitopes for a large number of bNAb$s$ (1–6). OD is therefore considered to be an important candidate for structure-based vaccine design. We previously reported the design of a nonglycosylated Escherichia coli–expressed outer-domain fragment immunogen (ODEC) that bound CD4 with micromolar affinity and elicited a modest neutralizing antibody response in rabbits (7). ODEC bound CD4 and bNAb b12 with ~200–250-fold weaker affinity (3–12 μM) as compared with full-length gp120 (10–30 nM). ODEC also showed a high tendency to aggregate during purification and storage. Sera elicited in ODEC-immunized animals without boosting with gp120 showed very weak anti-gp120 titers (7).

In this study, we rationally modified the initial ODEC design to generate several derivatives with improved yield, biophysical

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This article contains Figs. S1–S8.

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4 The abbreviations used are: bNAb, broadly neutralizing antibody; CD4bs, CD4-binding site; OD, outer domain; Env, envelope glycoprotein; MSA, multiple sequence alignment; Arg-βC, arginine hydrochloride; YSD, yeast surface display; RLU, relative luminescence units, SPR, surface plasmon resonance; HDX-MS, hydrogen–deuterium exchange coupled to mass spectrometry; BS, bridging sheet; iVSV, inactivated vesicular stomatitis virus; NHB, non-hydrogen–bonded; GdnHCl, guanidine hydrochloride; PDB, Protein Data Bank; sCD4, soluble CD4; TKBK, Tanford-Kirkwood model together with the Bashford-Karplus approximation.

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Table 1

| Protein | PDB Code |
|---------|----------|
| gp120   | 1G9M     |
| Core gp120 in CD4-bound conformation | |
| OD<sub>Ec</sub> | Initial design included most of the CD4 receptor binding site |
| BS-OD<sub>Ec</sub> | Conformationally variable region removed, size further minimized |
| Core gp120 in VRC01-bound conformation | |

Figure 1. Fragment-based immunogen design targeting outer domain of HIV-1 gp120 glycoprotein. A, structure of core gp120 (PDB code 1G9M) when complexed to CD4 (gray). gp120 can be broadly subdivided into inner domain (gray), outer domain (yellow), and bridging sheet regions (dark blue). CD4-binding site residues (CD4bs) are shown in red. B, part of the bridging sheet (blue) was included in the OD<sub>Ec</sub> design to maximize CD4 contacts. C, Focus on a bNAb (VRC01) epitope. D, structure of core gp120 when complexed to the CD4bs bNAb VRC01; coordinates are from the PDB code 3NGB (1, 2).

Design of HIV-1 outer-domain immunogens

The goal of this work was to generate fragment immunogens targeting conserved sites on the gp120 OD with high affinity. All OD immunogens described in this study could also bind to the germline-reverted version of the CD4bs-directed VRC01 antibody with nanomolar binding affinity. Immunization of rabbits with OD<sub>Ec</sub> followed by boosting with gp120 resulted in no detectable neutralization at week 22. However, the improved OD immunogens designed in this study could rapidly elicit sera with heterologous cross-clade neutralization of tier 1 viruses after 22 weeks. This study also provides insights into some of the biophysical correlates of improved immunogenicity.

Results

Initial OD design

The goal of this work was to generate fragment immunogens targeting conserved sites on the gp120 OD with high affinity. All outer-domain immunogens described in this study are based on a previously designed construct known as OD<sub>Ec</sub> (7). OD<sub>Ec</sub> design was focused on the CD4-binding site (CD4bs) of gp120 glycoprotein (Fig. 1, A and B; Table 1). In summary, CD4 (primary cellular receptor) interacting residues of gp120 were identified by calculating amino acid accessibilities in the presence and absence of CD4 using coordinates from the gp120–CD4 crystal structure (PDB code 1G9M (8)). Most CD4 interacting residues were found to be present in the OD and bridging sheet regions (Fig. 1A). Thus, an OD fragment (OD<sub>Ec</sub>) from residues 255 to 474 having most of the OD and a part of the bridging sheet was selected for bacterial expression (Fig. 1B). OD<sub>Ec</sub> retained ~70% of the CD4 epitope. It was devoid of the inner domain, part of the bridging sheet, V1V2 and V3 variable loops, and had 11 computationally guided and rationally designed hydrophobic to hydrophilic mutations at the interface of inner and outer domain to prevent aggregation and increase solubility, to minimize the chances of aggregation (7).

Purification and characterization of OD<sub>Ec</sub> refolded in the presence and absence of Arg-HCl

In the original study (7), OD<sub>Ec</sub> was purified by performing a 10-fold rapid dilution of denatured protein into PBS containing 1 mM EDTA, to reduce the GdnCl concentration from 6 to 0.6 M. This sudden refolding protocol was developed because OD<sub>Ec</sub> exhibits a very high tendency to aggregate upon slow removal of denaturant via dialysis. Sudden refolding of protein resulted in decreased but still significant precipitation during the refolding step and also during storage. Moreover, the refolded protein bound weakly with CD4 and bNAb b12, as compared with full-length gp120, indicating that it is not well-folded (7). To obtain a better-folded protein, we explored a number of variations in the refolding protocol and found that the inclusion of 0.5 M Arg-HCl resulted in reduced precipitation. Compared with the OD<sub>Ec</sub> protein that was refolded in the absence of Arg-HCl, the CD spectrum of the Arg-HCl–refolded protein showed a spectrum characteristic of a β-sheet (Fig. 3A). This is expected because, with the exception of one helix (A2), the outer domain is largely composed of a β-sheet structure.

Spr-binding studies with Arg-HCl–refolded OD<sub>Ec</sub>

We characterized the Arg-HCl–refolded OD<sub>Ec</sub> molecule for binding to 4-domain soluble CD4 (sCD4) and to the broadly neutralizing antibodies b12 and VRC01. VRC01 is a potent, broadly neutralizing antibody (1, 2) that binds to the CD4-binding site and shows cross-clade neutralization. As shown in Table 2, Arg-HCl–refolded OD<sub>Ec</sub> bound sCD4 with ~15-fold and b12 with ~80-fold improved affinity as compared with the protein refolded in the absence of Arg-HCl (7). OD<sub>Ec</sub> (Arg-HCl refolded) binds only ~5–15 times weaker to these ligands than core gp120 lacking V1V2 and V3 variable loops. Interestingly, Arg-HCl–refolded OD<sub>Ec</sub> also bound the antibody VRC01 with a K<sub>D</sub> of 359 ± 55 nm, which was about 15-fold weaker when compared with gp120 (Table 2).
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Table 1
Nomenclature and description of HIV-1 gp120 OD fragment constructs

| No. | Construct name | Description |
|-----|----------------|-------------|
| 1   | OD<sub>ECC</sub><sup>a</sup> | HIV-1 gp120 OD fragment from HXBc2 strain was codon-optimized for expression in E. coli (EC). This fragment consists of residues 255–474 of gp120 with 11 designed mutations to prevent aggregation. It lacks V1V2 and V3 variable loops, but retains V4 loop residues |
| 2   | ΔBS-OD<sub>ECC</sub> | OD<sub>Ecc</sub> lacking bridging sheet (BS) region residues from strands 20 and 21 |
| 3   | OD<sub>ECC</sub>Consensus | OD<sub>Ecc</sub> containing 27 consensus mutations |
| 4   | OD<sub>ECC</sub>CysV4 | OD<sub>Ecc</sub> containing S375W/T257C cavity-filling (CF) mutations |
| 5   | OD<sub>ECC</sub>CycV4 | OD<sub>Ecc</sub> containing an additional disulfide between residues 293 and 448 |
| 6   | OD<sub>ECC</sub>CycV4A | A cyclic permutant of ODEC, where new N and C termini are created at the V4 loop via deletion of residues 398–409, and the original N and C-termini were connected with a GSAG linker |
| 7   | OD<sub>ECC</sub>C1 (ΔG14–ΔBS-OD<sub>ECC</sub> + CF + SS) | A cyclic permutant of ODEC where new N and C termini are created at the V4 loop via deletion of residues 398–409, and the original N and C-termini were connected with a GSAG linker |
| 8   | OD<sub>ECC</sub>C2 (ΔG4–ΔBS-OD<sub>ECC</sub>Consensus + CF + SS) | A cyclic permutant of ODEC where new N and C termini are created at the V4 loop via deletion of residues 398–409, and the original N and C-termini were connected with a GSAG linker |

<sup>a</sup> Construct described in Ref. 7.
<sup>b</sup> Construct described in Ref. 23.
<sup>c</sup> Construct described in Ref. 18.

Table 2
Kinetic parameters for binding of various OD fragment constructs and full-length WT gp120 with bNAb VRC01, GL-VRC01, bNAb b12, and sCD4 as determined by SPR

| Ligand | Analyte | k<sub>on</sub>  | k<sub>off</sub>  | K<sub>d</sub> ± S.D. |
|--------|---------|---------------|----------------|---------------------|
| VRC01  | WT gp120| 3.3 × 10<sup>4</sup> | 8.7 × 10<sup>-4</sup> | 26 ± 5 |
|        | OD<sub>Ecc</sub> | (3.7 ± 0.3) × 10<sup>4</sup> | (1.3 ± 0.1) × 10<sup>-2</sup> | 359 ± 54 |
|        | ΔBS-OD<sub>ECC</sub> | 3.14 × 10<sup>4</sup> | (3.9 ± 0.7) × 10<sup>-2</sup> | 124 ± 22 |
|        | OD<sub>ECC</sub>Consensus | (1.8 ± 0.4) × 10<sup>5</sup> | (1.2 ± 0.1) × 10<sup>-2</sup> | 69 ± 18 |
|        | OD<sub>ECC</sub>CycV4 | (1.7 ± 0.7) × 10<sup>5</sup> | (2.5 ± 0.2) × 10<sup>-2</sup> | 16 ± 5 |
| GL-VRC01<sup>a</sup> | WT gp120 | NB | NB | NB |
|        | OD<sub>Ecc</sub> | 3.3 × 10<sup>5</sup> | 1.3 × 10<sup>-3</sup> | 4 ± 1 |
|        | ΔBS-OD<sub>ECC</sub> | (2.6 ± 0.2) × 10<sup>4</sup> | (1.8 ± 0.2) × 10<sup>-3</sup> | 71 ± 11 |
|        | OD<sub>ECC</sub>Consensus | (3.3 ± 1.6) × 10<sup>4</sup> | (5.6 ± 3.7) × 10<sup>-3</sup> | 16 ± 3 |
|        | OD<sub>ECC</sub>CycV4 | (2.6 ± 1.2) × 10<sup>5</sup> | (3.1 ± 0.2) × 10<sup>-2</sup> | 14 ± 5 |
| b12    | WT gp120 | 3.5 × 10<sup>4</sup> | 1.7 × 10<sup>-3</sup> | 49 ± 5 |
|        | OD<sub>Ecc</sub> | 1.7 × 10<sup>4</sup> | (2.7 ± 0.2) × 10<sup>-3</sup> | 153 ± 17 |
|        | ΔBS-OD<sub>ECC</sub> | (5.4 ± 1.0) × 10<sup>4</sup> | (3.5 ± 0.4) × 10<sup>-1</sup> | 690 ± 18 |
|        | OD<sub>ECC</sub>Consensus | (4.1 ± 0.2) × 10<sup>4</sup> | (2.6 ± 0.2) × 10<sup>-3</sup> | 64 ± 3 |
|        | OD<sub>ECC</sub>CycV4 | (8.3 ± 0.7) × 10<sup>4</sup> | (3.0 ± 0.2) × 10<sup>-2</sup> | 37 ± 1 |
| sCD4   | WT gp120 | 2.3 × 10<sup>4</sup> | 3.3 × 10<sup>-4</sup> | 15 ± 4 |
|        | OD<sub>Ecc</sub> | 1.9 × 10<sup>4</sup> | (4.2 ± 0.3) × 10<sup>-2</sup> | 219 ± 11 |
|        | ΔBS-OD<sub>ECC</sub> | (5.4 ± 0.3) × 10<sup>4</sup> | (2.8 ± 0.4) × 10<sup>-2</sup> | 528 ± 80 |
|        | OD<sub>ECC</sub>Consensus | (2.3 ± 0.3) × 10<sup>5</sup> | (5.3 ± 1.1) × 10<sup>-3</sup> | 230 ± 23 |
|        | OD<sub>ECC</sub>CycV4 | (7.3 ± 2.0) × 10<sup>4</sup> | (2.9 ± 0.5) × 10<sup>-3</sup> | 40 ± 5 |

<sup>a</sup> GL-VRC01 refers to germline reverted VRC01 (18); S.D. is standard deviation for the data from two independent experiments; NB means no detectable binding.

Designing next generation OD fragment immunogens

The OD<sub>ECC</sub> construct was further modified with an aim to improve its biophysical and binding properties. A part of the bridging sheet was included in the original OD<sub>ECC</sub> design, along with most of the outer domain of gp120 (Fig. 1B). In the CD4-bound conformation of gp120, the bridging sheet is made up of four anti-parallel β-strands (β3, β2, β21, and β20). β21- and β20-strands are close to the outer domain and are unlikely to be folded in the absence of the remaining two strands (8). However, these two strands were included in the original OD<sub>ECC</sub> design to retain certain critical CD4 contacts (Fig. 1B). The broadly neutralizing antibody VRC01 neutralizes around 91% of 190 viruses tested, with a geometric mean IC₅₀ of about 0.33 μg/ml VRC01 (2). VRC01 targets the CD4bs in the outer domain, it rotates about 40° with respect to CD4 and translates 6 Å away from the bridging sheet region, making the approach angle different from that of CD4. This causes the antibody to reduce its contact with the bridging sheet and the CD4-binding loop, while contacting the V5 loop in the proximal barrel of the outer domain (Fig. 1D) (1). As VRC01 makes minimal contacts with the bridging sheet, we removed the two bridging sheet strands present in the OD<sub>Ecc</sub> molecule. Bridging sheet residues from 423 to 434 were deleted, and residue 422 was connected to residue 435 using a Gly–Ala–Gly (GAG) linker in the ΔBS–OD<sub>ECC</sub> construct (ΔBS stands for bridging sheet deleted) (Fig. 1C). We hypothesized that apart from focusing the immune response to the VRC01 epitope, removal of this possibly unstructured bridging sheet region in ΔBS–OD<sub>ECC</sub> construct might also result in improved stability and other biophysical properties.

Methods for engineering proteins with improved stability can be classified as rational structure-based methods, directed evolution-based methods, and semi-rational sequence frequency-based methods (9). It is often difficult to predict stabilizing mutations solely using structure-based methods. Frequency-based methods make use of amino acid conservation among homologous proteins to identify possible beneficial mutations. Consensus protein design is based on the assumption that the frequency of a given amino acid at a particular position in a multiple sequence alignment (MSA) of related proteins is
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**Figure 2. Methodology for consensus-based design of an OD<sub>EC</sub> variant.** Consensus design makes use of amino acid conservation among homologous protein sequences to identify possible beneficial mutations. A total of 27 consensus mutations were introduced in the OD<sub>EC</sub> background to generate the OD<sub>EC</sub>Consensus construct.

Directly correlated with that amino acid’s contribution to protein stability or function. In other words, more conserved residues contribute more to the stability of a protein, and thus, replacing non-consensus residues with the consensus (most frequent) ones could result in stabilization (9–12). Using this consensus-based protein design approach, we made an OD<sub>EC</sub> variant named as OD<sub>EC</sub>Consensus (Fig. 2). Briefly, the most frequent amino acid at each position in a multiple sequence alignment of 814 HIV-1 B-subtype sequences was identified. Each residue in OD<sub>EC</sub> was mutated to the most frequent residue at that position in the MSA. Residues involved in interaction with CD4, bNAb b12, and bNAb VRC01 were identified using accessible surface calculations as described previously (13), and these were not mutated. In addition, 11 hydrophobic to hydrophilic mutations introduced in the original OD<sub>EC</sub> molecule to prevent aggregation were retained (Fig. 2). Finally, a total of 27 consensus mutations were introduced in the OD<sub>EC</sub> background (Fig. S1). Models for OD<sub>EC</sub> and OD<sub>EC</sub>Consensus constructs were made using the program MODELLER 9, version 1 (14, 15). TKBK electrostatic calculations confirmed that the consensus mutations have not introduced any unfavorable electrostatic interaction in OD (Fig. S2) (16, 17).

**Purification, biophysical characterization, and binding studies of new OD constructs (ΔBS-OD<sub>EC</sub> and OD<sub>EC</sub>Consensus)**

Both proteins were expressed and purified from *E. coli* for further characterization using Arg-HCl–assisted sudden refolding. The yield was about 3–4 mg/liter of the culture for OD<sub>EC</sub>Consensus (similar to OD<sub>EC</sub>) and ~20 mg/liter for ΔBS-OD<sub>EC</sub>. This drastic improvement in the yield for ΔBS-OD<sub>EC</sub> was attributed to the significant reduction in precipitation during the purification process, which could be due to the removal of the potentially unstructured bridging sheet region. SDS-PAGE studies confirmed that both the proteins were at least 90% pure. The expected masses for OD<sub>EC</sub>, ΔBS-OD<sub>EC</sub>, and OD<sub>EC</sub>Consensus were 23,033.0, 21,820.7, and 23,378.4 Da, respectively, whereas observed masses using ESI-MS were found to be 23035.0, 21,821.1, and 23,380.0 Da, respectively, confirming the identities of these proteins. The expected and observed masses correspond to the disulfide-formed oxidized mass of proteins.

CD spectra indicated that both the proteins are well-folded and predominantly in a β-sheet conformation as expected (Fig. 3, A and B). The fluorescence spectrum of these proteins showed an expected red-shift and a change in emission intensity upon denaturation, confirming that the proteins are likely to be folded with the burial of some tryptophan residues in the native state (Fig. 3C). Native proteins run on a C5 analytical reverse-phase column elute as a single peak, thereby showing that they exist as a homogeneous species in solution and not as a mixture of different disulfide-bonded isomers (18). The denatured, reduced proteins eluted at a different acetonitrile concentration than the native protein, re-confirming that native proteins are well-folded and oxidized (Fig. S3, A and B).

ΔBS-OD<sub>EC</sub> and OD<sub>EC</sub>Consensus were examined for binding to sCD4 and to the broadly neutralizing antibodies b12 and VRC01 on a Biacore 2000 machine. OD<sub>EC</sub>Consensus bound VRC01 with about 5–6 times better affinity than WT OD<sub>EC</sub>. Its binding to b12 was about 2–3 times better, whereas binding to CD4 was similar to OD<sub>EC</sub> (Table 2). ΔBS-OD<sub>EC</sub> bound VRC01 about 3–4 times tighter and to b12 and CD4 ~4–5-fold and ~2–3-fold weaker than corresponding values for WT OD<sub>EC</sub>. This reduction in the binding affinity for b12 and CD4 is expected for ΔBS-OD<sub>EC</sub> as the bridging sheet region contains important residues for interaction with b12 and CD4. The bridging sheet was deleted in this construct in an attempt to stabilize the molecule and to focus the design on the VRC01 epitope. A comparison of all kinetic parameters for binding is shown in Table 2. In an alternative attempt to improve the biophysical and binding properties of OD immunogens, we also designed OD constructs with additional disulfides or cavity-filling mutations as described below.

**Design of disulfide and cavity-filling mutants of OD<sub>EC</sub>**

Although naturally occurring disulfides are known to typically stabilize proteins, all designed disulfides do not necessarily result in stabilization. Certain stereochemical criteria need to be satisfied in order for a disulfide bond to form. We have previously reported the effect of the introduction of disulfides in different secondary structural elements in diverse proteins (19, 20). It was found that almost all naturally occurring cross-strand disulfides in anti-parallel β-strands are located at...
non-hydrogen–bonded (NHB) positions (19, 21). Subsequently, experiments on model proteins such as Top7, maltose-binding protein, and other periplasmic proteins showed that cysteines introduced at exposed NHB positions form disulfides spontaneously, whereas those engineered at hydrogen-bonded positions either remain in the reduced form or if they form a disulfide result in destabilization of the protein (19). Based on the above observations, we attempted to reduce the conformational flexibility of OD by engineering disulfides at an exposed NHB position (293–448) in an anti-parallel β-strand.

Residues 293 and 448 are present at the distal barrel of the outer domain, have high surface accessibility (59 and 42%, respectively), and a 4.61 Å distance between their Ca–Ca atoms. Both these residues were mutated to cysteines to introduce an additional disulfide bond in the ODSS molecule, which generated the ODSS construct (Fig. 4A; Table 1, no. 5).

Analysis of the crystal structure of gp120 bound to CD4 (PDB code 1G9M) shows that two prominent cavities exist in the binding region (22). A surface cavity of gp120 remains filled with water molecules and is surrounded on all sides by residues from gp120 and CD4. Another much deeper cavity extends into the interior of gp120. Phe-43 from CD4 plugs the opening of this cavity, and thus it is known as the Phe-43 cavity. Previously, a double mutant S375W/T257S of gp120 was shown to improve the binding affinity to CD4 without affecting b12 binding (23). In this study, we attempted to reduce the conformational entropy of OD and to stabilize it in a CD4-bound conformation by incorporating both these well-characterized cavity-filling mutations (T257S and S375W). This OD mutant was named as OD-Cavity filling (ODEC-CF) (Fig. 4A; Table 1).

Design of OD constructs with multiple designed mutations

Introducing various stabilizing mutations individually in the ODEC background did not result in achieving gp120-like affinity for bNAbs, so we decided to combine the various stabilizing mutations (described above) with the glycosylation site mutations earlier identified by our group (18). The first combined construct (ODEC-C1) was made by deleting the bridging sheet region coupled with the introduction of an additional disulfide (294–448) and cavity-filling mutations in the ΔG14-ODEC background (all 14 glycosylation sites present in the outer domain were mutated) (Table 1, no. 7) (18). In another combined construct, the additional disulfide and cavity-filling mutations were introduced in the bridging sheet–deleted ODEC-Cavity filling protein background. Four glycosylation sites close to the CD4bs (18) were also mutated in this construct to generate the ODEC-C2 construct (Table 1, no. 8).

Yeast surface display (YSD) of the next-generation OD immunogens

YSD was used as an initial screening system to characterize the newly designed OD immunogens. YSD allows rapid screening of a large number of mutants for binding with various ligands, thereby alleviating the need to express and purify the individual clones (Fig. S4) (24). Expression on the yeast surface has previously been shown to be directly correlated with the folding efficiency of the protein (25). In the YSD system, the protein of interest (OD fragment immunogens in this case) is displayed as a fusion to the adhesion subunit of the yeast agglutinin protein Aga2p, which attaches to the yeast cell wall through disulfide bonds to Aga1p (Fig. S4A). Expression of the Aga2p fusion is under the control of a galactose-inducible promoter on the yeast display plasmid pPNLS. Each yeast cell typically displays $1 \times 10^4$ to $1 \times 10^5$ copies of the protein. Protein surface expression can be detected by using fluorescently labeled antibodies against c-Myc and HA epitope tags followed by FACS. Binding of the surface-displayed molecule with a ligand can be detected by the addition of a fluorescently
labeled secondary antibody followed by FACS (Fig. S4A). This technique allows binding affinity determination in a surface display format without the need for purification and expression of each individual clone (Fig. S4B). YSD can also be used for rapid screening of mutant libraries (24, 26). All OD EC variants expressed well on the yeast surface. To assess the conformational integrity of surface-displayed OD molecules, their binding affinities with the conformation-specific VRC01 bNAb were determined using FACS (Table 3). The original OD EC molecule bound VRC01 with a \( K_D \) of \( \sim 400 \, \text{nm} \). ΔBS-OD EC and OD ECConsensus showed \( \sim 3-6 \)-fold higher affinity for VRC01 than OD EC (Table 3). Importantly, these constructs did not show measurable binding with the non-neutralizing mAb b6 as they lack regions of the b6 epitope. This improvement in affinity for the VRC01 bNAb shows that both these new constructs mimic the VRC01-binding epitope of gp120 better than the original OD EC construct, even on the yeast surface. The VRC01-binding affinities for OD EC, BS-ODEC, ΔBS-OD EC, and OD ECConsensus were already available from the SPR studies with the purified proteins (Table 2), and the fact that they match closely with the values obtained from YSD (Table 3) demonstrates the utility of YSD. Both OD EC,1 (Fig. S5) and OD EC,2 (Fig. S6) showed substantial improvement in the binding affinity for bNAb VRC01 (Table 3) with \( K_D \) values of about 3–4 nm. In comparison, yeast displayed core gp120 bound VRC01 with a \( K_D \) of \( \sim 24 \, \text{nm} \) (Table 3, no. 1).

Purification and characterization of OD EC,1 and OD EC,2

As both the combined constructs when displayed on the yeast surface showed significant improvement in VRC01 binding, we expressed and purified these two proteins from E. coli using Arg-HCl–assisted sudden refolding as has already been described for OD EC. The yield was about \( \sim 15-20 \, \text{mg/liter} \) of the culture for both the proteins.

Far-UV and near-UV CD spectra indicated that both the proteins are well-folded and predominantly in a \( \beta \)-sheet conformation (Fig. S8, A and B). The fluorescence spectra of these proteins showed an expected red-shift and a change in emission intensity upon denaturation, indicating that the proteins are likely to be folded with burial of some tryptophan residues in the native state (Fig. S7, A and B).

Isothermal urea denaturation studies were carried out to estimate stabilities of all the purified OD EC variants (OD EC, ΔBS-OD EC, OD ECConsensus, OD EC,1, and OD EC,2). The chemical unfolding of OD EC was not cooperative, whereas both ΔBS-OD EC and OD ECConsensus show a cooperative isothermal urea denaturation profile with apparent \( C_m \) values of 2.4 and 2.15 mM urea, respectively (Fig. 5). The free energy of unfolding at zero denaturant concentration (\( \Delta G^0(U) \)) was found to be \( \sim 1.6 \, \text{kcal/mol} \) for ΔBS-OD EC (\( m \) value = 0.85 kcal/mol/M) and \( \sim 2.23 \, \text{kcal/mol} \) for OD ECConsensus (\( m \) value = 0.95 kcal/mol/M). \( C_m \) is the concentration of denaturant at which 50% protein is unfolded, whereas the \( m \) value is proportional to the amount of surface area buried upon unfolding (27). OD EC,1 and OD EC,2 also showed a cooperative transition in urea-mediated denaturation with much higher apparent \( C_m \) values of 4.2 and 3.3 mM urea, respectively (Fig. 5). The free energy of unfolding at zero denaturant concentration (\( \Delta G^0(U) \)) was \( \sim 2.8 \, \text{kcal/mol} \) for OD EC,1 (\( m \) value = 0.67 kcal/mol/M) and \( \sim 2.53 \, \text{kcal/mol} \) for OD EC,2 (\( m \) value = 0.74 kcal/mol/M).
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Figure 5. Isothermal urea denaturation studies. The fraction unfolded (F_u) as a function of denaturant (urea) concentration monitored by fluorescence intensity is plotted. The denaturation was monitored at the wavelength maximum of the difference spectra of native (in 0 M urea) and denatured protein (in 6 M urea), typically in the range of 320–325 nm. Data for OD_EC Consensus, ΔBS-OD_EC, OD_EC C2, and OD_EC C1 were fitted to a two-state unfolding model (67, 68) resulting in a C_m of 2.2, 2.4, 3.3, and 4.2 M urea, respectively, whereas a C_m value for OD_EC (inset) could not be determined because of the absence of folded and unfolded baselines in the urea denaturation profile.

kcal/mol for OD_EC C2 (m value = −0.81 kcal/mol/s). A comparison of C_m and ΔG^0_u values coupled with YSD VRC01 binding measurements indicates that both the combined constructs OD_EC C1 and OD_EC C2 are more stable and better folded compared with the previous OD_EC mutants.

Design of a cyclic-permutant of OD_EC

Protein design largely relies on amino acid mutations to alter stability or function. Circular permutation of proteins is an attractive approach for protein design, as it allows manipulation of stability and folding with minimal perturbation of the three-dimensional structure (28–30). In cyclically permuted proteins, the original N and C termini are connected through a linker, whereas new N and C termini are generated by introducing a break elsewhere in the sequence (29). The OD_EC molecule is an artificially created fragment immunogen, and it is possible that the N- and C-terminal regions generated due to the removal of the inner domain as well as their neighboring regions are not correctly folded. The original OD_EC design consists of residues 255–474 from gp120, and thus the residues 255 and 474, respectively, form the N and C termini of the OD_EC molecule (Fig. 4B). The OD_EC fragment lacks V1V2 and V3 loops but retains V4 loop residues (7). The V4 loop is highly flexible, its function is not well-established, and it has a high tolerance for insertion, and a reasonable tolerance for deletions in the context of native, trimeric Env (31, 32), and it is therefore not considered to be a good vaccine target (33). Because of its high flexibility, the V4 loop region electron density is not visible in the gp120 crystal structure (PDB code 1G9M) (Fig. 4B) (8). To cyclically permute OD_EC, new N and C termini were created at the V4 loop region via deletion of residues 398–409. The old N and C termini of OD_EC were ~8 Å apart and connected via a GSAG (Gly–Ser–Ala–Gly) amino acid linker (Fig. 4B). The overall idea was to stabilize the regions close to the old N and C termini of OD_EC as they are important for the binding of various CD4bs ligands, like CD4 and bNAb VRC01, by making new N and C termini within the flexible V4 loop. We also hypothesized that removal of the immunodominant and flexible V4 variable loop might also result in an improved immune response toward the conserved CD4-binding site.

Purification, characterization, and SPR-binding studies of OD_EC CycV4

The protein was expressed and purified from E. coli using Arg-HCl–assisted sudden refolding as has been described for OD_EC. The yield was about 1–2 mg/liter. ESI-MS analysis was performed to confirm the identity of the protein. It appeared folded from far-UV CD (Fig. S8A), near-UV CD (Fig. S8B), and fluorescence spectra (Fig. S7C). OD_EC CycV4 was examined for binding to sCD4 and to the broadly neutralizing antibodies b12 and VRC01 on a Biacore 2000 machine. OD_EC CycV4 showed ~5-fold higher on-rates and ~5-fold slower off-rates for binding with bNAb VRC01, which resulted in ~20–30-fold improvement in the VRC01-binding affinity (K_d, 16 nM). OD_EC CycV4 also showed ~4–5-fold improved binding affinity for both CD4 and bNAb b12. The binding affinities of OD_EC CycV4 for CD4, bNAb b12, and bNAb VRC01 are comparable with that of full-length, glycosylated gp120 (Table 2). Bacterially expressed, glycan-free OD_EC CycV4 also bound germline-reverted VRC01 (GL-VRC01) with a K_d of ~10–20 nM, whereas, as expected, full-length glycosylated gp120 did not show any binding to GL-VRC01 (Table 2). All other OD constructs also show very high-affinity binding with GL-VRC01.

Prime/boost immunization studies with OD fragment immunogens

Immunization with gp120 primarily elicits non-neutralizing antibodies, in part because the immune response is not focused on conserved neutralization epitopes (Fig. 6A). Fragment immunogen design enables removal of non-neutralizing epitopes. However, it is possible that in such artificially designed fragment immunogens, the equilibrium between the folded and partially folded forms shifts toward the unfolded state (Fig. 6B). Hence, on immunization with less stable fragment immunogens, non-neutralizing antibodies against the unfolded state can still predominate. Protein stabilization can minimize the generation of non-neutralizing antibodies against the unfolded state and thus focus the immune response against conserved epitopes. After immunization with fragment immunogens, a full-length better-folded protein (for e.g. gp120) could be used as a boost to selectively amplify the cross-reactive antibodies, resulting in increased amounts of CD4-binding site antibodies (Fig. 6C). Therefore, we used a prime/boost strategy to study the potential of OD immunogens to elicit neutralizing antibodies. In each group, an OD_EC variant was used as the priming immunogen at weeks 0 and 4 followed by an Env variant as the boosting immunogen at weeks 12 and 20 (Table 4). In addition to WT JRFL gp120, two other Env derivatives were used. IVSV-JRFL is inactivated vesicular stomatitis virus displaying Env ect-
odomain and membrane-proximal external region joined to the vesicular stomatitis virus G transmembrane and cytoplasmic domains on its surface (34). V1cycP gp120 molecule is a trim-eric cyclic permutant of gp120, made by introducing new N and C termini at the V1 loop (at amino acids 144–142) while con-necting the original N and C termini with a short linker. A human cartilage matrix protein trimerization domain was also added at the newly created N and C termini with a short linker. The V1cycP gp120 molecule (30, 35) provides the best boost for inducing high anti-gp120 titers. In summary, the ELISA results indicated that the ODECCycV4 and ODECC2 are the best primes for eliciting sera with high anti-gp120 titers. Moreover, both of them are significantly improved relative to the original ODEC molecule, which could not elicit any measure-able gp120 titers on its own, without two gp120 boosts (Table 4).

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|----------------------------------------|

**Figure 6. Schematic representation of the prime/boost strategy.** A, conventional gp120 prime/gp120 boost immunization, where relatively lower levels of CD4-binding site targeting neutralizing antibodies (encircled antibodies represented in orange) are produced. Because of the presence of immunodominant variable regions as well as a significant fraction of partially folded/unfolded molecules, most of the immune response against monomeric gp120 is non-neutralizing (antibodies against the variable regions are represented in blue) in nature. B, fragment immunogen carrying the CD4-binding site exists in equilibrium between folded and partially folded/unfolded forms; therefore, on boosting with gp120, the non-neutralizing antibodies against the unfolded state (red) can still predominate. C, mutations stabilizing the folded conformation of the fragment immunogen decrease the antibody response against the unfolded state (red) and increase the response against the CD4bs (orange). Upon boosting with full-length gp120, the cross-reactive antibodies (encircled antibodies represented in orange; directed toward the CD4-binding site, and antibodies represented in blue; directed toward regions other than the CD4bs) are selectively amplified, resulting in increased amounts of CD4-binding site antibodies.

**Table 4:**

| OD design | Average week 6 anti-gp120 titers |
|-----------|---------------------------------|
| BS-ODEC   | /H11011 103                  |
| ODEC      | /H11011 104                  |
| ODECC1    | /H11011 310                  |
| ODECConsensus | /H11011 103     |
| ODECC2    | /H11011 104                  |
| ODECCycV4 | /H11011 103                  |

By comparing all the groups boosted with monomeric gp120 protein, the OD priming immunogens can be arranged in increasing order of their ability to induce anti-gp120 titers (week 22) as follows: ΔBS-ODEC < OD_{EC} < OD_{EC,C1} < OD_{EC,Consensus} < OD_{EC,C2} < OD_{EC,CycV4}. Similarly, a comparison of week 22 anti-gp120 titers for the three groups primed with BS-ODEC but boosted with differ-ent Env derivatives indicated that the V1cycP gp120 previously designed by us (30, 35, 36) provides the best boost for inducing high anti-gp120 titers. In summary, the ELISA results indicated that the OD_{EC,CycV4} and OD_{EC,C2} are the best primes for eliciting sera with high anti-gp120 titers. Moreover, both of them are significantly improved relative to the original OD_{EC} molecule, which could not elicit any measure-able gp120 titers on its own, without two gp120 boosts (Table 4).
Pseudoviral neutralization assay in TZM-bl cells

Pre-immune sera and week 22 sera from all the groups were tested in pseudoviral neutralization assays in TZM-bl cells. The week 22 sera from multiple groups show moderate to good neutralization of MN.3 (clade B, tier 1) and MW965.26 (clade C, tier 1) viruses (Table 5). As expected from the anti-gp120 titers, group 6 (∆BS-ODECprime, V1cycP gp120 boost) showed the best neutralization with ID_{50} values as high as 10^4 for these two viruses. Neutralization titers (ID_{50}) are the sample dilution at which relative luminescence units (RLU) were reduced by 50% compared with RLU in virus control wells after subtraction of background RLU in cell control wells. Groups 2 and 7 (∆BS-ODEC prime, monomeric gp120 boost) exhibited modest neutralization of MN.3 (clade B, tier 1) and MW965.26 (clade C, tier 1) viruses with ID_{50} values up to 395 (Table 5). Group 3 (ODECConsensus prime, monomeric gp120 boost) showed the best neutralization with higher median neutralization ID_{50} values for ODEC2 and ODECConsensus immunogens over ODEC in terms of their capacity to generate cross-reactive anti-gp120 sera (Table 4) and neutralizing antibodies (Table 5).

Consistent with the anti-gp120 titer values, group 5 (ODEC,C2 prime) and group 8 (ODEC,CycV4 prime) showed the best neutralization with higher median neutralization ID_{50} values compared with other groups boosted with monomeric gp120. The tier 1 neutralization ID_{50} values for ODEC,C2 and ODEC,CycV4-immunized animals were significantly higher as compared with the ∆BS-ODEC-immunized animals (p values were 0.0070 and 0.0425, respectively, two-tailed non-parametric Mann-Whitney test at 95% confidence interval). The tier 1 neutralization ID_{50} values for ODEC2 and ODEC,CycV4-immunized animals were qualitatively better than the ODEC-Consensus-immunized animals; however, the differences were not statistically significant at the 95% confidence interval (p value = 0.3170, two-tailed non-parametric Mann-Whitney test). Surprisingly, group 4 (ODEC,C1 prime) did not show any significant neutralization. This study indicates that ODECConsensus is a better prime than ∆BS-ODEC and the V1cycP gp120 is the best molecule for the boost, among the candidates tested (Table 5).
### Table 5

**ID<sub>50</sub> values for virus neutralization with pre-immune and week 22 sera**

| Group No; Animal ID; (Prime: Boost) | SVA-MLV (Neg. Ctrl) | MN.3 (1/B) | MW965.26 (1/C) | JRFL (2/B) | Ce1176_A3 (2/C) |
|------------------------------------|---------------------|------------|----------------|------------|----------------|
| 1; 246-249; pre-immune pooled      | <20                 | <20        | <20            | <20        | <20            |
| 1; 248; (OD<sub>PCEC</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 1; 247; (OD<sub>PCEC</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 1; 249; (OD<sub>PCEC</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 2; 1357-1360; pre-immune pooled    | <20                 | <20        | <20            | <20        | <20            |
| 2; 1357; (ABS-OD<sub>PCEC</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 2; 1358; (ABS-OD<sub>PCEC</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 2; 1359; (ABS-OD<sub>PCEC</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 2; 1360; (ABS-OD<sub>PCEC</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 3; 1361-1364; pre-immune pooled    | <20                 | <20        | <20            | <20        | <20            |
| 3; 1361; (OD<sub>PCEC-Consensus</sub>: gp120) | 193                 | 171        |                 | <20        | <20            |
| 3; 1362; (OD<sub>PCEC-Consensus</sub>: gp120) | 395                 | <20        | <20            | <20        | <20            |
| 3; 1363; (OD<sub>PCEC-Consensus</sub>: gp120) | 49                  | <20        | <20            | <20        | <20            |
| 3; 1364; (OD<sub>PCEC-Consensus</sub>: gp120) | 240                 | <20        | <20            | <20        | <20            |
| 4; 1365-1368; pre-immune pooled    | <20                 | <20        | <20            | <20        | <20            |
| 4; 1365; (OD<sub>PCEC:C1</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 4; 1366; (OD<sub>PCEC:C1</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 4; 1367; (OD<sub>PCEC:C1</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 4; 1367; (OD<sub>PCEC:C1</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 5; 1369-1372; pre-immune pooled    | <20                 | <20        | <20            | <20        | <20            |
| 5; 1369; (OD<sub>PCEC:C2</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 5; 1370; (OD<sub>PCEC:C2</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 5; 1371; (OD<sub>PCEC:C2</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 5; 1372; (OD<sub>PCEC:C2</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 6; 1373-1376; pre-immune pooled    | <20                 | <20        | <20            | <20        | <20            |
| 6; 1373; (ABS-OD<sub>PCEC:VsCyCp</sub> gp120<sup>a</sup>) | <20                 | <20        | <20            | <20        | <20            |
| 6; 1374; (ABS-OD<sub>PCEC:VsCyCp</sub> gp120<sup>a</sup>) | <20                 | <20        | <20            | <20        | <20            |
| 6; 1375; (ABS-OD<sub>PCEC:VsCyCp</sub> gp120<sup>a</sup>) | <20                 | <20        | <20            | <20        | <20            |
| 6; 1376; (ABS-OD<sub>PCEC:VsCyCp</sub> gp120<sup>a</sup>) | <20                 | <20        | <20            | <20        | <20            |
| 7; Pre immune pooled               | <20                 | <20        | <20            | <20        | <20            |
| 7; 1377; (ABS-OD<sub>PCEC:IVSV-JRFL</sub>)<sup>a</sup> | 28                  | 27         | <20            | <20        | <20            |
| 7; 1378; (ABS-OD<sub>PCEC:IVSV-JRFL</sub>)<sup>a</sup> | <20                 | <20        | <20            | <20        | <20            |
| 7; 1379; (ABS-OD<sub>PCEC:IVSV-JRFL</sub>)<sup>a</sup> | <20                 | <20        | <20            | <20        | <20            |
| 7; 1380; (ABS-OD<sub>PCEC:IVSV-JRFL</sub>)<sup>a</sup> | <20                 | <20        | <20            | <20        | <20            |
| 8; 1381-1384; pre-immune pooled    | <20                 | <20        | <20            | <20        | <20            |
| 8; 1381; (OD<sub>PCEC:CycV4</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 8; 1382; (OD<sub>PCEC:CycV4</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 8; 1383; (OD<sub>PCEC:CycV4</sub>: gp120) | <20                 | <20        | <20            | <20        | <20            |
| 8; 1384; (OD<sub>PCEC:CycV4</sub>: gp120) | <20                 | 623        | <20            | <20        | <20            |

* The ID<sub>50</sub> values are the serum dilution at which relative luminescence units (RLUs) were reduced 50% compared to virus control wells (no test sample). For each virus, the tier category/clade is mentioned in parentheses. The color code indicates the following: 20 < ID<sub>50</sub> < 50 (green, weak neutralization); 50 < ID<sub>50</sub> < 200 (yellow, moderate neutralization); ID<sub>50</sub> > 200 (red, strong neutralization). White and gray colors indicate the absence of measurable neutralization.

<sup>a</sup> V1cycP gp120 (hCMP (144-142) V1cyc-JRCSF gp120) used in this study was previously designed in our laboratory by the introduction of the hCMP trimerization domain at the N terminus of the cyclically permuted gp120 (35).

<sup>b</sup> Inactivated vesicular stomatitis virus (iVSV) expressing JRFL Env ectodomain and MPER joined to the VSV G transmembrane (TM) and (CT) domains on the surface.
bodies as compared with previously designed OD_{EC} and newly designed ΔBS-OD_{EC} and OD_{EC}Consensus immunogens.

**SPR-binding studies with OD_{EC}C1 and OD_{EC}C2**

Both OD_{EC}C1 and OD_{EC}C2 were selected for the immunization studies because they bound VRC01 with very high affinity when displayed on yeast and demonstrated an increased resistance to urea denaturation. However, sera from animals primed with OD_{EC}C1 failed to show any neutralization in the TZM-bl cell assays (Table 5). This was surprising because the week 22 anti-gp120 titers elicited by OD_{EC}C1 were comparable with the other groups (Table 4). To understand the reason for this discrepancy, we did SPR-binding studies with these two proteins (Table 6). In agreement with the yeast surface display results (K_{D} ~4 nm), purified OD_{EC}C2 protein bound VRC01 with a very high affinity (K_{D} = 16 ± 8 nm). However, purified OD_{EC}C1 bound VRC01 with a much lower affinity (K_{D} = 200 ± 70 nm) than it did on the yeast surface (K_{D} ~3.4 nm) (Table 3). It is important to note that the OD_{EC} protein purified without Arg-HCl refolding bound CD4bs ligands with 3–12 μM affinity (7). The VRC01-binding affinity for the yeast-displayed OD_{EC} protein was ~400 nm. The inclusion of Arg-HCl in the refolding buffer resulted in purified OD_{EC} achieving a VRC01 affinity comparable with that on the yeast surface (Tables 2 and 3). These data suggest that the protein purification protocol used in this study does not result in properly refolded OD_{EC}C1 protein, and therefore VRC01-binding affinity is much lower for protein purified from *E. coli* as compared with protein displayed on yeast.

**HDX-MS studies**

We next performed hydrogen–deuterium exchange (HDX) studies coupled to MS to probe the conformational flexibility of both the initial OD_{EC} construct and of those OD immunogens that elicited measurable neutralization in TZM-bl assays. In these HDX experiments, protonated protein is placed in D_{2}O (pH ~6.8). Under these conditions, exchangeable protons, such as amide protons and other protons attached to polar/charged atoms, are replaced by deuterium. Protons present in side chains exchange very fast, but hydrogens linked to buried amide functional groups exchange on time scales that can be measured and hence can provide information on protein structure and dynamics. The exchange of protons with deuterium is probed by analyzing the increase in mass of the protein as a function of time. The rate of exchange depends on various factors such as pH, temperature, and the accessibility of the exchangeable hydrogen to solvent (37). Buried hydrogens within a protein can exchange through local/global unfolding of the protein. As shown in Fig. 7A, OD_{EC} showed a very fast rate of exchange in the first couple of minutes, with about 130 backbone amide hydrogens getting deuterated in that time. From 1 to 20 min, there was a slow phase, during which another 5–7 deuteriums are added. However, the mass obtained after 20 min of exchange was identical to that obtained after 1–2 h of exchange at 37 °C (saturation). This indicated that all the backbone amide hydrogens were exchanged within 20 min (Fig. 7A). It is expected that a conformationally stable domain/sub-domain in a protein should show slower hydrogen exchange kinetics, with some protons remaining protected even after hours of exchange. These experiments show that although OD_{EC} appears to be well-structured by CD and shows a prominent thermal transition, it is liable to hydrogen–deuterium exchange and therefore is dynamically flexible (Fig. 7A). Both ΔBS-OD_{EC} and OD_{EC}Consensus proteins show similar exchange kinetics with near saturation observed near around 20 min, although 4–6 protons exchanged between 20 min to 1 h, whereas OD_{EC} attains complete saturation at 20 min (Fig. 7, A–C). This indicates a small improvement in the local structural rigidity for both the designs. Surprisingly, OD_{EC}C2, which elicited sera with moderate pseudoviral neutralization appears to have faster hydrogen exchange kinetics as compared with OD_{EC} (Fig. 7D). In case of OD_{EC}CycV4, a fast exchange of about 115 protons is seen as in OD_{EC}. A near saturation value is reached at about 20 min. However, a slow exchange of about four protons is seen up to 2 h, after which saturation is reached. Thus, cyclic permutation results in a significantly slower exchange of some protons (Fig. 7E). In all cases, around 30% of protons show rapid back exchange and hence could not be probed. HDX-MS profiles of corresponding regions in full-length gp120 follow similar exchange profiles as outer-domain-derived immunogens for most of the peptides (38). For most of the peptides in the outer domain, about 80% exchange is complete by 30 min with the exception of peptides 282–286 and 436–443, which show less than 20% exchange even after 4 h (38). In case of the BG505 SOSIP.664 gp140 trimer, most of the outer-domain region peptides show exchange of about 75% protons in 30 min with the exception of 284–287 and 446–453 peptides, which show less than 20% exchange even after 4 h (39). The slightly faster exchange seen for our constructs compared with full-length gp120 and trimERIC gp140 may be due to truncation of these fragment

### Table 6

| Ligand   | Analyte       | k_{on}       | k_{off}     | K_{D}   |
|----------|---------------|--------------|-------------|---------|
|          |               | s^{-1}        | s^{-1}       | nm      |
| VRC01    | WT gp120      | 3.3 × 10^{4}  | 8.7 × 10^{4} | 26 ± 5  |
|          | OD_{EC}C1     | (3.7 ± 0.3) × 10^{4} | (1.3 ± 0.1) × 10^{-2} | 359 ± 54 |
|          | OD_{EC}C2     | (7.5 ± 2.3) × 10^{4} | (1.4 ± 0.1) × 10^{-3} | 200 ± 70 |
|          | (5.0 ± 3.5) × 10^{4} | (6.7 ± 1.8) × 10^{-4} | 16 ± 8   |
| GL-VRC01 | WT gp120      | NB           | NB          | NB      |
|          | OD_{EC}C1     | 3.3 × 10^{2}  | 1.3 × 10^{3} | 4 ± 1   |
|          | OD_{EC}C2     | (5.2 ± 2.9) × 10^{3} | (1.7 ± 0.6) × 10^{-3} | 400 ± 170 |
|          | (3.4 ± 3.7) × 10^{4} | (5.2 ± 0.7) × 10^{-4} | 48 ± 10  |
immunogens from a large protein, leading to greater solvent accessibility of several regions.

Discussion

Monomeric gp120, when used as an immunogen, has failed to elicit broadly neutralizing antibodies against HIV-1 (40–45). This, in part, is due to the very high conformational flexibility of the gp120 molecule. In an effort to reduce the size of the immunogen and focus the immune response to a smaller number of epitopes, an outer-domain immunogen (OD\textsubscript{EC}) lacking the flexible loops and the inner domain was designed previously by us (7). It was expressed in E. coli to prevent glycosylation and to increase yield. The protein was found to bind CD4 and the neutralizing antibodies b12 weakly ($K_D$ of 3–12 \(\mu\)M) but did not bind the non-neutralizing antibodies b6 and F105. The far-UV CD spectrum of OD\textsubscript{EC} indicated that it had considerable secondary structure, although the large dip near 210 nm also indicated that there was a substantial amount of an unfolded population. Therefore, we searched for alternative ways of refolding OD\textsubscript{EC}. In this work, we modified the purification protocol for OD\textsubscript{EC} by including Arg-HCl in the refolding buffer. Arg-HCl is a well-known stabilizing osmolyte, known to help in the refolding of proteins by destabilizing expanded states like the unfolded state of a protein (46, 47). In contrast to the OD\textsubscript{EC} refolded in PBS, the CD spectrum of the Arg-HCl–refolded protein showed a characteristic spectrum of a $\beta$-sheet consistent with its known structure in the context of gp120. The Arg-HCl–refolded OD\textsubscript{EC} bound CD4 and b12, respectively, with $\sim$15 and 80 times higher affinity than the OD\textsubscript{EC} refolded in the absence of Arg-HCl. OD\textsubscript{EC}, when displayed on the yeast surface, binds VRC01 with a $K_D$ of 400 nM; OD\textsubscript{EC} refolded in the presence of Arg-HCl also binds VRC01 with a similar affinity indicating that this in vitro refolded molecule is probably as well-folded as the one folded in vivo in a eukaryotic system. However, these affinities were still $\sim$5–15 times weaker than those of core gp120 for VRC01, and therefore we attempted to stabilize OD\textsubscript{EC} further through mutations.

Two such modified OD\textsubscript{EC} immunogens ($\Delta$BS-OD\textsubscript{EC} and OD\textsubscript{EC}Consensus), when purified using the improved refolding protocol, bound various CD4bs ligands with $K_D$ values of $\sim$100 nm. However, their binding affinities for various CD4bs ligands were still significantly lower than the full-length gp120 molecule. Additionally, the binding of $\Delta$BS-OD\textsubscript{EC} with CD4 and bNAb b12 was reduced significantly, due to the removal of the bridging sheet. Hydrogen exchange mass spectrometric studies showed these molecules are still highly flexible. To further reduce the conformational flexibility of OD\textsubscript{EC} cavity-filling and disulfide mutations were introduced. As binding to CD4 normally leads to a large change in entropy in gp120, previous studies on full-length gp120 had shown that filling the Phe-43 cavity using S375W/T257S mutations reduces this entropy change and thereby leads to a higher affinity for CD4 (22, 23). In this work, we tested these mutations in the OD\textsubscript{EC} background. An OD\textsubscript{EC} mutant with these two cavity-filling mutations (OD\textsubscript{EC}CF), when displayed on the yeast surface, showed $\sim$6-fold improvement in the VRC01-binding affinity. An OD\textsubscript{EC} disulfide mutant (OD\textsubscript{EC}SS) with an additional disulfide between NHB pairs 293 and 448 was also designed and expressed on the yeast surface. It showed a marginal improvement in binding with VRC01 bNAb.

As all the above designs ($\Delta$BS-OD\textsubscript{EC}, OD\textsubscript{EC}Consensus, OD\textsubscript{EC}CF, and OD\textsubscript{EC}SS) showed some improvement in binding with VRC01 bNAb, we decided to combine these mutations with the glycosylation-site mutations identified earlier by our group because these mutations also improve binding for CD4bs ligands (18). The first combined construct (OD\textsubscript{EC}C1) had mutations at all the 14 outer-domain glycosylation sites, cavity-filling mutations, an additional disulfide, and was devoid of the bridging sheet region. Another combination was made (OD\textsubscript{EC}C2) by the introduction of four glycosylation-site muta-

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**Figure 7. HDX-MS profiles.** Proteins were diluted 20-fold in D\textsubscript{2}O (pH 6.8), allowed to exchange at 20 °C, and quenched with 2% formic acid. Repr-

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**Image:**

- HDX-MS profiles: A) OD\textsubscript{EC}, charge state $+25$, 90% of the protons exchange in 30 s, followed by a slower exchange of about 5–7 protons. Exchange is complete after 20 min (1200 s). B) $\Delta$BS-OD\textsubscript{EC}, charge state $+24$, a fast exchange is seen for about 80% of the protons in 30 s followed by a slower exchange of about 5–7 protons from 20 min to 1 h (3600 s) when the exchange is complete. C) OD\textsubscript{EC}Consensus, charge state $+25$, a fast exchange is seen for about 80% of the protons in 30 s, followed by a slow exchange of about 6–8 protons from 20 min to 1 h when the exchange is complete. D) OD\textsubscript{EC}C2, charge state $+18$, a fast exchange is seen for about 95% of the protons in 30 s followed by exchange of the remaining protons by 20 min when the exchange is complete. E) OD\textsubscript{EC}CycV4, charge state $+24$, a fast exchange is seen for about 90% of the protons in 1 min followed by a slow exchange of about 4 protons from 20 min to 2 h when the exchange is complete.
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tions, consensus mutations, cavity-filling mutations, and a disulfide mutation in the ΔBS-OD_{EC} background (Table 1). Both of these combined constructs, when displayed on the yeast surface, bound bNab VRC01 with very high affinity (3–4 nM \( K_D \)). E. coli purified OD_{EC}C1 and OD_{EC}C2 showed a cooperative transition in urea-mediated denaturation with an apparent \( C_m \) of 4.2 and 3.26 m urea, respectively. This is a significant improvement over ΔBS-OD_{EC} and OD_{EC}Consensus immunogens, which show apparent \( C_m \) values of 2.4 and 2.1 m urea, respectively. These results demonstrate that combining glycosylation site mutations with rational stabilizing mutations results in significantly improved affinity and chemical stability, even when the molecules are bacterially expressed and lack glycans.

Designing circular permutants of proteins is an attractive approach for protein design as it allows manipulation of protein topology without changing its amino acid composition or the three-dimensional structure. In an alternative attempt to stabilize the OD_{EC} design, we made a cyclic permutant of OD_{EC}. E. coli expressed, cyclic-permutant OD_{EC}CycV4 protein bound CD4, bNab b12, and bNab VRC01 with affinities comparable to that of full-length WT gp120. It bound VRC01 with 16 ± 5 nM \( K_D \), whereas the original OD_{EC} construct with native connectivity binds VRC01 with 359 ± 54 nM \( K_D \). As we have not introduced any additional stabilizing mutations in the OD_{EC}CycV4 constructs, it indicates that cyclic permutation indeed helped in a better structural organization of the CD4bs. HDX-MS studies also showed that among all the OD_{EC} variants tested, OD_{EC}CycV4 was the slowest to undergo exchange, indicating increased rigidity.

Broadly NAbS against HIV-1 show a very high level of affinity maturation, and it has been proposed that targeting the germ-line form of these bNAbs is a pre-requisite for generating bNAbs (48–51). Full-length glycosylated gp120 fails to engage the germ-line forms of bNAbs and is thus considered a poor immunogen for the activation of B-cells carrying germ-line variants of bNAbs. Apart from binding mature VRC01 with nongeminal \( K_D \), OD_{EC}CycV4 also showed high-affinity binding (~10 nm \( K_D \)) with its germ-line variant (GL-VRC01), indicating that it is a promising immunogen. It is important to note that all our OD immunogens are produced from E. coli and are thus glycan-free. All these OD immunogens bind germ-line-reverted VRC01 with very high affinity. Even the original OD_{EC} immunogen without any additional stabilizing mutation binds GL-VRC01 with ~4 nm \( K_D \), whereas full-length glycosylated gp120 does not show measurable binding with GL-VRC01, indicating that glycosylation is one of the main barriers in germ-line recognition of broadly neutralizing antibodies. However, this hypothesis needs to be tested for the germ-lines of other CD4bs targeting bNAbs.

The OD designs with the best \textit{in vitro} properties were tested in a rabbit immunization study. Following monomeric gp120 boosts, groups primed with OD_{EC}C2 and OD_{EC}CycV4 showed the highest anti-gp120 titers relative to the rest of the molecules tested (OD_{EC}BS, OD_{EC}Consensus, and OD_{EC}C1).

The week 22 sera elicited by OD_{EC}C2 and OD_{EC}CycV4 immunization could neutralize tier 1 viruses from clade B as well as clade C with a higher potency as compared with the ΔBS-OD_{EC} and OD_{EC}Consensus immunized groups boosted with monomeric gp120. Clade B viruses are more prevalent in North America and Europe, whereas clade C viruses are more common in Africa and Asia. This cross-clade neutralization elicited by our immunogens is encouraging because the week 22 sera from OD_{EC}-immunized animals could not show any measurable neutralization. Our results indicate that improvement in stability and affinity helps in faster elicitation of anti-gp120–neutralizing antibodies. However, we could not observe any tier 2 neutralization.

We expect that the combination of OD_{EC}C2/OD_{EC}CycV4 prime and V1cycP gp120 boost, possibly coupled with a longer immunization protocol, should further increase the neutralization potency and breadth of the sera. A longer immunization study is required because anti-HIV-1 broadly neutralizing antibodies typically take a long time to develop, as has been observed in primate experiments (52) and patients (2, 53, 54) and also in one of our recent studies (55).

Various studies attempting to focus the immune response to the outer domain of HIV-1 Env have been carried out previously. An OD construct (OD1) based on YU2 gp120 strain contained residues 252–482, retained V1V2 and V3 variable loops, was glycosylated, and was expressed in \textit{Drosophila} S2 cells, but the sera from rabbit immunizations failed to neutralize homologous YU2 virus (56). Mouse immunization studies with clade-C OD as a fusion of human IgG1 Fc domain did not elicit any neutralizing response (57, 58). In another study two OD immunogens (monomeric and trimeric) based on the group M consensus sequence were used to elicit heterologous tier 1 neutralizing responses, although higher neutralization titers for all viruses were seen with sera immunized with monomeric gp120 (59). A mammalian cell-expressed cyclically permutated OD derivative (eOD-Base) lacking glycans at Asn-276 and Asn-463 was evolved using Rosetta design followed by random mutagenesis and yeast display library screening to form the eOD-GT6 mutant, which bound GL-VRC01 with 44 nm \( K_D \) values, and several other GL antibodies with high affinity while retaining binding with the mature bNAbs. A modified variant of eOD-GT6 (eOD-GT8 60-mer) (60) was used to immunize knock-in mice modified to express the germ-line-reverted heavy chain of VRC01 (VH1–2*02) under the control of a mouse VH promoter. eOD-GT8 60-mer immunized mice could activate VRC01 class precursors, whereas BG505 trimers failed to do so, possibly because these trimeric molecules do not bind germ-line variants of VRC01. In another study, the immune responses were primed with eOD-GT6 60-mer (a high-affinity germ-line VRC01 binder) and were subsequently boosted with a series of different immunogens with a decreased affinity toward germ-line VRC01 compared with mature VRC01. However, the sera could only neutralize viruses lacking glycans near the CD4bs. Whether these germ-line VRC01 binders induce VRC01 lineage antibodies when IGHV1–2*02HC and VRC01LC are present at physiological frequencies remains to be tested (61). Recently, an OD construct based on subtype C strain 1084i lacking V3 loop regions, having stabilizing disulfides and a cavity-filling mutation (OD3), was able to elicit a tier 1 and a weak tier 2 neutralizing response in guinea pig immunization studies (62). Our OD immunogens also lack the V3 loop, and the neutraliza-
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Materials and methods

Construct descriptions

A summary of OD construct details is provided in Table 1. The new OD immunogens are described in greater detail under “Results.”

Electrostatic calculations using Tanford-Kirkwood model

An accessibility corrected TKBK was used for calculating electrostatic interactions between charged residues in the immunogens (65). A program implementing the algorithm for these calculations as described in Ibarra-Molero et al. (17) was kindly provided by Dr. J. M. Sanchez-Ruiz (Facultad de Ciencias, Departamento de Química Física, Spain). Classically, this theory was developed for calculating charge–charge interactions assuming a spherical shape for any protein, and ionizable amino acids were represented by point charges smeared over the spherical surface of the protein. All globular proteins were assumed to have an average specific volume of 0.72 ml/g. Hence, the radius of any protein sphere can be calculated based on the molecular weight of the protein. The solvent was assumed to be a continuum with a high dielectric constant of 78 and protein had a low dielectric constant of 2 or 4. Subsequently, when 3D structures of proteins became available, the interaction energy between a pair of amino acids was corrected for by their surface accessibility in the 3D structure, as the energy for a pair of residues on the surface of a protein would be lower relative to that at a buried position due to dielectric screening by the solvent (14). A positive value for the charge–charge interaction energy (E_{int}) indicates unfavorable interaction and vice versa.

Cloning, expression, and protein purification

The E. coli codon-optimized gene sequences of our designed immunogens were synthesized (GenScript) and cloned into the pET28a(+) vector (Novagen) between the NdeI and BamHI sites with an N-terminal His tag. The proteins were overexpressed in E. coli BL21(DE3) cells and purified from the insoluble fraction of the cell culture lysate. Briefly, a single colony of E. coli BL21(DE3) transformed with the plasmid of interest was inoculated into 5 ml of Luria-Broth (LB). The primary culture was grown overnight at 37 °C. One liter of LB was inoculated with 1% of the primary inoculum and grown at 37 °C until an A_{600} of ~0.6 – 0.8. Cells were then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside and grown for another 8 – 12 h at 37 °C. Cells were harvested at 3500 × g and resuspended in 35 ml of phosphate-buffered saline (PBS) (pH 7.4), containing 100 μM phenylmethylsulfonyl fluoride and 0.2% Triton X-100. The cell suspension was lysed by sonication on ice and centrifuged at 15,000 × g. The supernatant was discarded, and the pellet was resuspended in 35 ml of 0.2% Triton X-100, PBS (pH 7.4), sonicated again on ice, and subjected to centrifugation at 15,000 × g. The pellet was solubilized in 40 ml of 6 M GdnCl in PBS (pH 7.4) overnight at room temperature. The solution was centrifuged at 15,000 × g for 30 min. The supernatant was bound to 3 ml of nickel-nitrilotriacetic acid beads (GE Healthcare) and washed twice with 25 ml of 50 mM imidazole containing 6 M GdnCl in PBS (pH 7.4), and finally the denatured protein was eluted with 6 M GdnCl in PBS (pH 7.4) containing 500 mM imidazole at room temperature.

The first four eluted fractions (each 5 ml) were pooled together and then rapidly diluted 10-fold with PBS containing 1 mM EDTA to reduce the denaturant concentration from 6 to 0.6 m. The resulting solution was again concentrated back to the original volume in an Amicon concentrator. This was followed by desalting into PBS (pH 7.4) containing 1 mM EDTA using a HiTrap desalting column (Amersham Biosciences) to remove the remaining denaturant. The desalted protein was concentrated to a final concentration of 0.5 mg/ml, flash-frozen in liquid nitrogen, and stored in aliquots at −80 °C. The yield was determined by densitometry analysis from SDS-PAGE using standard proteins of known concentrations.

This purification protocol was further modified to include Arg-HCl in the refolding buffer. Briefly, the first three elution fractions (5 ml each) were pooled together and rapidly diluted 10-fold with 0.5 M Arg-HCl (SRL, 99% purity) in PBS containing 1 mM EDTA at 4 °C to reduce the denaturant concentration from 6 to 0.6 m. The resulting solution was again concentrated back to the original volume in an Amicon concentrator. This was followed by desalting into PBS (pH 7.4) containing 1 mM EDTA using a HiTrap desalting column (Amersham Biosciences) to remove the remaining denaturant. The desalted protein was concentrated to a final concentration of 0.5 mg/ml, flash-frozen in liquid nitrogen, and stored in aliquots at −80 °C. The protein yield was determined by densitometry analysis from SDS-PAGE using standard proteins of known concentrations. All proteins in this study were purified using a similar protocol and their identity was confirmed by ESI-MS.
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Far UV-circular dichroism (CD)

Far UV-CD spectra were recorded on a Jasco J-715C spectropolarimeter flushed with nitrogen gas. The concentration of all protein samples was ~5–10 μM and buffer used was PBS (pH 7.4). Measurements were recorded at 25 °C in a 1-mm path length quartz cuvette with a scan rate of 50 nm/min, a response time of 4 s, and a bandwidth of 2 nm. Each spectrum was an average of three scans. Mean residue ellipticities were calculated as described previously (66). Buffer spectra were also acquired under similar conditions and subtracted from protein spectra, before analysis. The experimental data plotting and fitting were done using SigmaPlot™ for Windows™ scientific graphing software.

Near UV-CD

Near UV-CD (tertiary CD) spectra were recorded on a Jasco J-715Cs spectropolarimeter flushed with nitrogen gas. Measurements were done over a wavelength range of 250 to 300 nm at 25 °C with 10–80 μM protein in PBS buffer (pH 7.4) with a 1-cm path length cuvette, a scan-rate of 10 nm/min, a response time of 8 s, and a bandwidth of 2 nm. Data reported were averaged over five scans and were corrected for buffer signals.

Fluorescence spectroscopy

All fluorescence spectra were recorded at 25 °C on a JASCO FP-6300 spectrofluorometer. The concentration of protein used was 1 μM either in PBS (pH 7.4) or in the presence of 6 M GdnCl in PBS (pH 7.4). The excitation wavelength was fixed at 280 nm, and emission was recorded from 300 to 400 nm. The excitation and emission slit widths were 3 and 5 nm, respectively. Each spectrum was an average of three consecutive scans. Buffer spectra were also acquired under similar conditions and subtracted.

Isothermal urea denaturation

Isothermal urea denaturation studies were carried out for all the proteins by monitoring their tryptophan fluorescence as a function of urea concentration as described previously (67). The final concentration of protein used was ~1–3 μM in PBS buffer (pH 7.4). Proteins were incubated with varying concentrations of urea (0–6 M) until equilibria were established. Urea concentrations were estimated by measurement of refractive indices of various urea dilutions. The denaturation was monitored at the wavelength maximum of the difference spectra of native (in 0 M urea) and denatured protein (in 6 M urea). This was typically in the range of 320–325 nm. Fluorescence spectra were acquired on a JASCO FP-6300 spectrofluorimeter with an excitation wavelength at 280 nm. The excitation and emission slit widths were 3 and 5 nm, respectively. The data obtained was fit to a two-state model as described previously (67, 68). Experimental data plotting and fitting were done using SigmaPlot™ for Windows™ scientific graphing software.

HPLC

20 μg of the native protein in PBS (pH 7.4) was injected into a Discovery C5 analytical column (150 × 4.6 mm, 5 μm particle size) (Supelco), whereas for reduced samples, the 20 μg of protein were incubated with 4 M GdnCl in PBS (pH 7.4) and 5 mM DTT at 37 °C, prior to injection. Proteins were eluted with a gradient of 5–95% acetonitrile containing 0.1% formic acid at a flow rate of 2% per min.

Binding affinity measurements using SPR

All SPR experiments were performed with a Biacore 2000 (Biacore, Uppsala, Sweden) optical biosensor at 25 °C. 800–900 resonance units of 4-domain CD4, b12, VRC01, b6, or F105 were attached by standard amine coupling to the surface of a research-grade CM5 chip. A sensor surface (without CD4 or an antibody) that had been activated and deactivated served as a negative control for each binding interaction. Different concentrations of analytes were passed across each sensor surface in a running buffer of PBS (pH 7.4) containing 0.005% P20 surfactant. Both binding and dissociation were measured at a flow rate of 30 μl/min. In all cases, the sensor surface was regenerated between binding reactions by one to two washes with 4 M MgCl₂ for 30 s at 30 μl/min. Each binding curve was corrected for non-specific binding by subtraction of the signal obtained from the negative-control flow cell. The kinetic parameters were obtained by fitting the data to the simple 1:1 Langmuir interaction model using BIA EVALUATION 3.1 software.

HDX-MS

For HDX-MS experiments, 50 pmol of ODSC or an ODSC variant protein (1.2 μg in 3 μl) at 20 °C was diluted 20 times in D₂O (Sigma) to a final volume of 100 μl and incubated for varying amounts of time. The pH of the resulting solution was ~6.8. The reaction was quenched by addition of 2 μl of 10% formic acid to a final concentration of 0.2% (pH 2.3) and immediately used for LC ESI-MS. A NanoAcquity UPLC system from Waters was used. The auxiliary solvent (which pushes the sample from the loop into the trap column) used was 0.1% formic acid in water, whereas the binary solvents were water and acetonitrile, each containing 0.1% formic acid. The sample was typically desalted using a 3-min acetonitrile gradient from 3 to 97%. For acquiring mass spectra, a Waters Q-Tof mass spectrometer was used. The capillary voltage was set to 3 kV, whereas the desolvation and source temperatures were set to 200 and 80 °C, respectively. The data were collected in positive ion mode.

One spectrum was collected/s. Spectra were combined in the MassLynx software and processed using the following parameters: (i) background subtraction: polynomial order, 20; below curve, 40%; (ii) smoothing: smooth windows (channels), 40; the number of smooths, 2; smooth mode, Savitzky Golay; (iii) centroid: minimum peak width at half-height, 2; centroid mode, top 80%. Following this, the spectrum was deconvoluted either automatically or manually by choosing two consecutive peaks at a time. For deuterated samples, a high standard deviation of 2–3 Da was usually obtained in the deconvoluted mass due to heterogeneity in the amount of deuterium incorporated in different molecules in the mixture; hence, the masses of individual charge states were monitored. The difference in mass of the protein obtained in the protonated solvent, as compared with that obtained after incubation in D₂O, was calculated for different time points.
Cloning of OD immunogens into a yeast surface display vector

Genes for various OD immunogens (ODEC, ABS-ODEC, ODCCConsensus, ODCC-CF, ODCC-SS, ODCC-C1 and ODCC-C2) were synthesized by GenScript (USA). These gene products were PCR amplified with pPNLS specific primers and individually recombined into SfiI digested linearized pPNLS vector by homologous recombination in the EBY100 strain of S. cerevisiae (24, 26, 69).

Yeast surface display of OD variants

pPNLS vector contains an AGA2p fusion at the N terminus of the surface-displayed protein along with two epitope tags, HA (YPYDVPDYA) and c-Myc (EQKLISEEDL), for detection (69). All constructs were displayed on the cell surface of Saccharomyces cerevisiae strain EBY100 using a standard protocol (26). Briefly, EBY100 cells were transformed with pPNLS plasmids containing genes for OD immunogens, and colonies were grown in glucose-containing liquid SCAA media (pH 4.0) until mid-log phase at 30 °C, followed by induction in galactose-containing SGCAA (pH 4.0) media at 20 °C for 16–24 h.

Strains, antibodies, and reagents

Monoclonal antibody (mAb) IgG-b12, gp120, and sCD4 were obtained from the Neutralizing Antibody Consortium of the International AIDS Vaccine Initiative (IAVI), New York. The plasmids for VRC01 and GL-VRC01 antibodies were kindly provided by Prof. John Mascola (National Institutes of Health). Both these antibodies were purified from HEK 293T mammalian cells using lentil–lectin affinity chromatography.

Yeast surface display and flow cytometric analysis

The surface expression for yeast displayed proteins was monitored using a chicken anti-c-Myc antibody (1:400 dilution) (Life Technologies, Inc.) as the primary antibody and anti-chicken Alexa Fluor 488 (Life Technologies, Inc.) (1:400 dilution) as the secondary antibody. Binding to anti-HIV-1 bNAb VRC01 was determined using goat anti-human phycoerythrin (YPYDVPDYA) and c-Myc (EQKLISEEDL), for detection of the surface-displayed protein along with two epitope tags, for detection (69). After each step, cells were washed with the labeling buffer (0.5% BSA in PBS (pH 7.4)). Cells were analyzed either on an Accuri C6 cytomter (BD Accuri Cytometers, Ann Arbor, MI), Canto II cytomter (BD Biosciences), or an ARIA III Instrument (BD Biosciences).

Prime/boost immunization studies

Immunization studies were carried out at Abexome Biosciences, Bangalore (SP1011-029), for group 1 and Covance Immunological Services in Denver, PA (C0129-13), for groups 2–8, using different OD fragment immunogens as the prime and different Env derivatives as the boost. Each group had four rabbits (New Zealand White, female, 3–5 kg). The studies followed animal research guidelines and were approved by the appropriate IACUC. All groups were primed at weeks 0 and 4 and boosted at weeks 12 and 20. Adjulex™ (Advanced Bio adjuvants LLC, Omaha, NE) was used as an adjuvant in this study. 20 μg of protein was used for intramuscular injections, and sera were collected at weeks 6, 14, and 22, heat-inactivated, and stored in aliquots.

Neutralization assay

Neutralizing antibody activity was measured in a standard in vitro assay known as TZM-bl neutralization assay as described previously (70, 71). Neutralization titers (ID50) are defined as the sample dilution at which RLUs are reduced by 50% as compared with RLUs in virus control wells after subtraction of background RLUs in cell control wells.

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