1
2 # Virus-like Particles Identify an HIV V1V2 Apex-Binding Neutralizing Antibody that Lacks a Protruding Loop
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29   **SUMMARY**

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31   Most HIV-1-specific neutralizing antibodies isolated to date exhibit unusual characteristics that

32   complicate their elicitation. Neutralizing antibodies that target the V1V2 apex of the HIV-1

33   envelope (Env) trimer feature unusually long protruding loops, enabl them to penetrate the HIV-1

34   glycan shield. As antibodies with loops of requisite length are created through uncommon

35   recombination events, an alternative mode of apex binding has been sought. Here, we isolated a

36   lineage of Env apex-directed neutralizing antibodies, N90-VRC38.01-11, using virus-like particles

37   and conformationally stabilized Env trimers as B cell probes. A crystal structure of N90-VRC38.01

38   with a scaffolded V1V2 revealed a binding mode involving side-chain to side-chain interactions

39   that reduced the distance the antibody loop must traverse the glycan shield, facilitating V1V2

40   binding via a non-protruding loop. The N90-VRC38 lineage identifies a solution for V1V2apex

41   binding that provides a more conventional B cell pathway for vaccine design.

42

43   **KEY WORDS**

44

INTRODUCTION

Neutralizing antibodies (NAbs) are likely to be a key component of effective HIV-1 vaccine immunity (Mascola and Montefiori, 2010). NAbs interfere with HIV-1 infection by binding to envelope (Env) spikes (comprised of gp120/gp41 trimers) on virion surfaces, thereby blocking receptor engagement and/or membrane fusion (Overbaugh and Morris, 2012). The glycan shield encasing these trimers helps the virus to evade NAbs, in part because carbohydrates are self-antigens to which antibody (Ab) responses are likely regulated by tolerance. Nevertheless, most, if not all, broadly neutralizing antibodies (bnAbs) make some glycan contacts upon native Env trimer binding (Stewart-Jones et al., 2016).

HIV-1 vaccine candidates can induce autologous NAbs but largely fail to induce NAbs against other circulating (tier 2) strains (Crooks et al., 2015; de Taeye et al., 2015; McCoy and Weiss, 2013). In contrast, cross-reactive NAbs develop in ~50% of HIV-1 infections (Doria-Rose et al., 2010; Hraber et al., 2014). Isolating monoclonal NAbs from such donors affords opportunities to understand how they develop and may be useful as vaccine blueprints (Burton and Hangartner, 2016).

Monoclonal bnAbs fall into several epitope clusters that, together, cover most of the trimer surface (Pancera et al., 2014; Ward and Wilson, 2015). The consistent features in different bnAbs suggest that a limited number of repertoire solutions can effectively tackle this complex antigen (Kwong and Mascola, 2012; Mascola and Haynes, 2013). One group of bnAbs targets the gp120 V1V2 loop at the trimer apex and includes PG9/16, CH01-04, CAP256.VRC26.01-33, and PGT141-145/PGDM1400-1412 (Andrabi et al., 2015; Bonsignori et al., 2011; Doria-Rose et al., 2015; Doria-Rose et al., 2014; Gorman et al., 2016; McLellan et al., 2011; Moore et al., 2011; Pancera et al., 2010; Sok et al., 2014; Walker et al., 2011; Walker et al., 2009). These NAbs exhibit unusually long (>24 amino acid (AA) by Kabat numbering) anionic third heavy chain complementarity determining regions (CDRH3) that are often tyrosine sulfated (excluding CH01-04) and project outward to penetrate the glycan shield and contact underlying protein. Abs with long CDRH3s naturally occur at low frequency due to a need for unusual recombination events and their regulation by tolerance (Briney et al., 2012a; Briney et al., 2012b; Haynes et al., 2012). Therefore, one goal of ongoing bnAb discovery is to identify NAbs with common repertoire features that are amenable to vaccine design.

NAb recovery efforts have taken two approaches. One involves high throughput screening of memory B cell micro-cultures that identified known V1V2-directed bnAbs (Bonsignori et al., 2011; Doria-Rose et al., 2014; Walker et al., 2011; Walker et al., 2009). A second approach is to

80   label desirable memory B cells with fluorescent "baits", followed by single cell sorting and RT-

81   PCR (Doria-Rose et al., 2015; Kong et al., 2016; Sok et al., 2014). Here virus-like particles (VLPs)

82   that present trimers in a natural membrane context (Crooks et al., 2015; Crooks et al., 2011; Hicar

83   et al., 2010) to probe memory B cells of a donor whose serum exhibited broad neutralization. We

84   recovered a NAb lineage of moderate potency and breadth, N90-VRC38.01-11, that bound the

85   V1V2 apex via an average length, non-protruding CDRH3revealing a new vaccine .

RESULTS

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      To develop B cell probe, we co-transfected plasmids encoding SIV Gag, Env, Rev and Gag-GFP (Figure S1). Concentrated supernatants were protease digested, resulting in "GFP-Trimer VLPs". SIV Gag and Gag-RFP plasmids were co-transfected to make "RFP-bald VLPs" as a counterstain. VLP labeling was verified using monoclonal Ab (mAb)-expressing Ramos cells and primary seronegative B cells (Figure S1). VLPs were then used to probe donor N90 B cells, whose serum neutralizes in a pattern that not track with known bnAbs (Georgiev et al., 2013; Huang et al., 2014) (Figure S2A). Negatively-selected N90 memory B cells were stained and gated for singlets, CD3⁻, CD8⁻, CD14⁻, CD19⁺, IgG⁺, GFP⁺ and RFP⁻ (Figure S). The extended positive shoulder imparted by trimer VLP suggested specific labeling. We selected cells shown as green dots (Figure 1B). Despite a study in which VLPs recovered lipid binding Abs (Hicar et al., 2010), RFP-bald VLP counterstaining was weak. The difference could relate to the higher VLP dose used for staining that allowed stringent washing (Figures S1C and S1D), but may also relate to the relatively dim RFP signal (Figure S1F). Following cell lysis, heavy and light chain variable segments were RT-PCR amplified, sequenced and aligned. We prioritized clones with NAb features: i) >10% divergence from germline VH (heavy chain variable domain) nucleotide sequence, ii) long CDRH3 loops, iii) repeated recovery and iv) insertion and deletion mutations. Two related clones, VRC38.01 and VRC38.02 (dark green dots in Figure 1B), were identified.

      To recover VRC38 relatives, a second sort used PE-labeled BG505 SOSIP gp140 trimers (a near native Env ectodomain mutant derived from the BG505 strain) as a positive probe. As VRC38.01 neutralizes the HIV-1 strain YU2 but does not bind to gp140F (a non-native, uncleaved Env ectodomain bearing a C-terminal foldon trimerization tag) derived from the same strain by ELISA (Figures S2D and S2E), APC-labeled YU2 gp140F trimers were used as a counterstain. PE⁺APC⁻ sorting (Figure S) recovered 9 more variants, VRC38.03-11 (dark red dots in Figure 1C), all of which show little or no gp140F binding (Figures S S).

      VRC38 variants had a 16 AA CDRH3 and a 10 AA CDRL3, diverged 15-20% nucleotide from germline VH3-13 (Figure 1D) and neutralized 3-42% of a multi-clade 31-member virus panel (Figure 1E). Geometric mean IC50 titers (GMT) against sensitive viruses were 0.076-1.62 µg/ml. Although VRC38.09 was not as broad as VRC38.01, it neutralized one VRC38.01-resistant virus (X1632.S2.B10) (Figures 1E and S2A). We paired 3 VRC38 heavy chain orphans from the second sort (Figure S2B) with the VRC38.01 light chain, one of which, VRC38.14, neutralized a VRC38.01-resistant strain (ZA012.29). Combining all lineage members, breadth against the 31-

120   member virus panel was 48%. The broadest clone, VRC38.01 neutralized 30% of a panel of 208
121   Env-pseudoviruses () and did not react with host antigens, as measured byHep2 (human epithelial
122   type 2) staining (Figure S). Despite the donor's North American origin and clade B infection (Wu
123   et al., 2012), VRC38 NAbs potently neutralized several clade A and AE viruses.

124   Neutralization profiles (Figure S2A) suggested that VRC38-like NAbs were not a dominant
125   contributor to serum breadth. Several viruses were sensitive to the N90 serum but not to VRC38
126   clones. Purified N90 serum immunoglobulin G (IgG) did not compete with VRC38.01 for binding
127   to JR-FL NFL trimers (Figure S2C), further suggesting low prevalence (<1μg/ml; Figure S2C).
128   Overall, the VRC38 lineage was in the donor, exhibited 15-20% germline divergence, modest
129   neutralization potency and breadth and no autoreactivity.

130

131   **VRC38 argets the V1V2-trimer pex**

132   VRC38.01's ability to neutralize the YU2 strain (Figure 1E), despite poor YU2 gp140F
133   binding (Figures S and S), suggested a quaternary epitope. By ELISA, VRC38.01 competed
134   strongly with V1V2 bnAbs for binding trimer VLPs and, to a lesser extent, with glycan-V3 bnAb
135   PGT121 (Figure 2A). Reciprocally, V1V2 bnAbs, PGT121 and to a modest extent, glycan-V3
136   bnAb PGT125 inhibited VRC38.01 binding (Figure SA). Neutralization of the JR-FL strain without
137   a E168K mutation (Figure 1E), suggested a distinct V1V2 binding mode. nlike VRC38.01, this
138   mutation was crucial for other V1V2 NAbs, and in some cases (PG9, PG16 and CH01) N189A
139   mutation was also needed for full sensitivity (Figure SB) (Doores and Burton, 2010).

140   To assess quaternary epitope dependency, we compared binding to monomeric gp120
141   and trimeric SOSIPs of sensitive strains, Q23.17 and DU156.12. Like PGT145, VRC38.01 bound
142   to trimers but not monomers, whereas glycan-V3 bnAb PGT128 bound to forms of Env (Figure
143   2B). PG9 bound to DU156.12 but not Q23.17 gp120 (Figure 2B). Further analysis revealed
144   VRC38.01 and PG9 bound to gp120 monomers from several neutralization-sensitive strains
145   (Figure 2C). Together, this data suggests that exhibit quaternary epitope preference, but that this
146   is not a strict requirement for binding.

147   The footprint of VRC38.01 Fab (fragment, antigen-binding) was examined on BG505
148   SOSIP trimers by negative-stain electron microscopy (EM, Figure 2D). 2D class averages showed
149   binding near the trimer apex. PG9 and CAP256.09, for which 3D reconstructions available
150   (EMDB-2241 and EMDB-5856, respectively) appeared to straddle gp120 protomers, binding
151   adjacent to the 3-fold axis at a slightly off vertical angle. 2D views of complexes with Fabs
152   PDGM1400 or VRC38.01 were insufficient for 3D modeling. Nevertheless, class averages
153   suggested footprints indicated in Figure 2D. VRC38.01's footprint was farther from the 3-fold axis

154   and showed little protomer overlap. Overall, this data showed that VRC38.01 targets the V1V2
155   apex with a quaternary epitope preference.
156
157   **VRC38.01 inds "off center" at ess than 3:1 toichiometry**
158   V1V2 bnAbs typically bind the trimer with a stoichiometry of 1:1, in part because their
159   footprints straddle gp120 protomers. 2D class averages of VRC38.01 Fab-trimer complexes by
160   EM indicated 1 or 2 Fabs per trimer in ~85% or ~15% of cases, respectively (Figure 2D). Using
161   VRC38.01 IgG, rather than the Fab, increased the proportion of complexes containing two Abs to
162   65% (Figure 2D), perhaps due to the increased avidity of two Fab arms; however, as the
163   crystallizable (Fc) of the IgGs was unresolved, it was unclear whether both Fabs originated from
164   the same IgG in the images. Related to this point, VRC38.01 IgG was much more potent (17- to
165   170-fold) than its monovalent Fab, unlike PG9 (1- to 3.5-fold higher) and PGT145 (1- to 12-fold
166   higher) (Figure SA). Furthermore, real time binding of VRC38.01 IgG to BG505 SOSIP trimers
167   showed >7-fold stronger binding over the Fab (Figure SB), also supporting the idea that
168   VRC38.01 IgG may engage trimers with both Fab arms simultaneously.
169   stoichiometry was investigated by three further methods. First, we checked bnAb-
170   mediated native trimer mobility shifts. 2G12 bound progressively to trimers with increasing
171   concentrations, providing a reference ladder (Figure SC). Saturating concentrations of all V1V2
172   NAbs bound at one copy per trimer (Figure SC). In a second approach, we measured binding
173   kinetics by biolayer interferometry (BLI). A standard curve was first generated with various trimer-
174   NAb complexes of known ligand stoichiometries at saturation (Figure SD). By interpolation, we
175   inferred that ~2 copies of VRC38.01 (Fab or IgG) occupied the trimer (Figure SD). In a third
176   approach, we performed isothermal titration calorimetry (ITC) to assess VRC38.01 Fab binding
177   to the BG505 native, flexibly linked (NFL) trimer, resulting in an estimate of N=2.26 (Figure SE).
178   Previously, one copy PG9 Fab resulted in N=0.6–0.8 (Julien et al., 2013; Sanders et al., 2013), 2
179   copies of PGT151 Fab resulted in N=1.3 (Blattner et al., 2014) and 3 copies of PGT121 or 2G12
180   Fabs binding per trimer gave N=2.3–2.4 (Sanders et al., 2013). Thus, an N value of 2.26 suggests
181   that 2-3 VRC38.01 Fabs bind per trimer.
182   he discrepancies above may relate to assay , with negative-stain EM and BN-PAGE
183   emphasizing binding at equilibrium and BLI and ITC emphasizing on rate and maximum binding.
184   Another contributing factor may be that the trimer strain and form differed between assays. Similar
185   discrepancies were previously noted (Lee et al., 2015). Collectively, our data suggest sub-trimer
186   (Figures 2D and S).
187

188 **VRC38.01 argets V1V2 pex lycans ia a on-rotruding oop**

189 The off-center VRC38.01 footprint (Figure 2D), and its ability to neutralize viruses lacking

190 a K168 residue (Figure SB) suggested a unique binding mode. To obtain atomic , a V1V2-1FD6

191 scaffold. For reference, we first determined the unliganded VRC38.01 Fab structure to 1.6 Å, then

192 solved the complex structure to 3.5 Å by molecular replacement. Refinement yielded an R_{work}/R_{free}

193 of 17.3/20.2 and 23.8/28.2 for the unliganded Fab and co-crystal structures, respectively (Figures

194 3A-C, S, Table S). No significant binding-induced conformational changes were observed

195 (Figures 3B, 3C and S5A). Overall, these structures revealed V1V2 loop binding via protein-

196 protein side-chain interactions with strands A, B and C and extensive N156 and N160 glycan

197 binding (Figures 3 and S).

198 The V1V2 domain formed a 5-stranded ß-barrel as for near-native BG505 Env trimers

199 (Pancera et al., 2014; Stewart-Jones et al., 2016), with a root-mean-square deviation (rmsd) of

200 1.1 Å across the ß-strand residues (Figure 3C). Extensive VRC38.01-*N*-linked glycan contacts

201 had buried surface areas of 465 Å^2 and 573 Å^2 for N156 and N160 glycans, respectively, each

202 contributing a hydrogen bond (Figure 3D-E). Mutations revealed that VRC38.01 was critically

203 dependent on glycan N156 and heavily depended on glycan N160 (Figure S). Minor contacts

204 were observed between the light chain and glycan N133 (Figures 3A and 3E). Protein-protein

205 contacts accounted for 1,135Å^2 of buried surface area. Seven hydrogen bonds were observed,

206 six from the heavy chain and one from the light chain (Figure S).

207 Alignment of the complex to trimeric Env via the V1V2 (Figure 3F) confirmed the off-center

208 footprint seen by EM (Figure 2D). Although direct VRC38.01 inter-protomer interactions were not

209 observed, the Fab aligned closely to the N160 glycan of a neighboring gp120 protomer and the

210 9-residue disordered loop between positions N185 and S187 (Figure S). To probe these potential

211 quaternary interactions, we engineered a glycan into the scaffold to mimic the neighboring N160

212 (scaffold insert position is shown in Figure 4I). Although electron density observed for this

213 engineered glycan was distal from the VRC38.01 epitope (Figure S), N160 positioning is not

214 precise, we rule out this interaction on the trimer. However, VRC38.01 binds monomeric gp120

215 of several strains, N160 glycan binding is probably not essential. The nine AAs following residue

216 185 in gp120 are disordered in available trimer structures and proximal to the framework 3 and

217 CDRH1 of VRC38.01 (). However, mutagenesis suggested that this region did not affect

218 VRC38.01, although CH01 and PGT145 were adversely affected by I184A mutations in the

219 WITO.33 and BG505 strains, respectively, proximal to this region (Figure S). Analysis of the

220 docked model in Figure 3F suggested that VRC38.01 Fabs could potentially occupy one HIV-1

221 trimer with no clashes (overlap cutoff >0.4Å; Figure S). The sub-saturating VRC38.01

222 stoichiometry (Figures 2D and S) may therefore stem from glycan and/or trimer structural
223 heterogeneity (Liao et al., 2013). Based on the distance of the modeled Fab C-termini, VRC38.01
224 is unique in being potentially able to use both IgG arms to engage the trimer. The large differences
225 VRC38.01 IgG and Fab neutralization potency (Figure SA) and trimer affinity (Figure SB) lend
226 indirect support this idea. In summary, the cocrystal reveals N156 and/or N160 glycan-
227 dependence, but also light chain contacts and a possibility to engage the trimer with both IgG
228 arms.
229
230 **VRC38.01 akes idechainidechain ontacts at the V2 pex** VRC38.01 exhibits a near charge-
231 neutral 16 AA CDRH3, unlike other V1V2 NAbs that exhibit unusually long (>24 AA) anionic
232 CDRH3s (Figure 4A) (Andrabi et al., 2015; Bonsignori et al., 2011; Doria-Rose et al., 2015;
233 Gorman et al., 2016; McLellan et al., 2011; Walker et al., 2011; Walker et al., 2009). Although
234 CH01 has greater breadth (53%) than its clonal variant CH03 (42%), we analyzed CH03 (AA
235 sequence identity 83% in the VH and VL regions), due to the available V1V2-complex structure
236 (Gorman et al., 2016). A comparison to normal Ab repertoire sequences (Shi et al., 2014) revealed
237 CDRH3 length within normal range, but other V1V2 NAbs outliers (Figure 4B). VRC38.01 exhibits
238 a near-neutral CDRH3 charge, is common in the repertoire (Figure 4C). In contrast, other V1V2
239 NAb CDRH3 loops are negatively charged due to prevalent Glu and Asp residues and sulfated
240 tyrosines (Figure 4A, 4E and 4F), which interact with the positively charged strand C (Andrabi et
241 al., 2015; Bonsignori et al., 2011; Doria-Rose et al., 2015; Gorman et al., 2016; McLellan et al.,
242 2011; Walker et al., 2011; Walker et al., 2009). Like other V1V2 NAbs, VRC38.01 diverged from
243 its germline (18%; Figure 4A and 4D).
244   Inspection of CDRH3-V1V2 contacts revealed VRC38.01 bind a site more distal from the
245 trimer axis than PG9 and CH03 (Figure 4G), consistent with its footprint (Figure 2D). Like other
246 V1V2 NAbs, VRC38.01 bound the N156 and N160 glycans of strand B (Figure 4G and S), but
247 made protein-protein contacts solely through Ab side-chain interactions, in contrast to PG9 and
248 CH03made extensive main-chain parallel strand-strand interactions with strand C residues 167-
249 171 proximal to the inter-protomer interface (Figures 4H and S). The CDRH3 and CDRH1 loops
250 of VRC38.01 hydrogen bonded side chains of strands A, B and C, including two salt bridges
251 formed with residues K168 and K171 of strand C and heavy chain residues D31 and E97,
252 respectively (Figure 4H). This side chain-dependent binding shortens the distance the CDRH3
253 loop must penetrate the glycan shield to reach underlying protein, inga short CDRH3 (Figure 4G-
254 I).
255

256 VRC38.01 ontacts lycans N156 and N160 and trand C but lashes with lycan N130

257   VRC38.01 eutralized 30% of a diverse panel of 208 pseudoviruses (Figure S and) at an

258 IC50<50 µg/ml, comparable to HJ16 and better than 2G12 (Figure SA). VRC38's GMT against

259 the 62 sensitive viruses (0.46 µg/ml) was lower than that of CH03 (0.54 µg/ml), 2G12 (1.56 µg/ml),

260 b12 (1.00 µg/ml), 2F5 (1.63 µg/ml), and 8ANC195 (0.88 µg/ml) and was the same as HJ16 (0.46

261 µg/ml) (Figure S6A). VRC38.01 exhibited higher activity against 27 subtype A viruses (), where

262 breadth and median IC50 were 52% and 0.043µg/ml, respectivelyclade A recombinants. Overall,

263 VRC38.01 exhibited modest potency and breadth.

264   Some bnAbs, including PG9 and PGT145, often plateau at <100% neutralization, a

265 phenomenon attributed in part to Env glycan heterogeneity (Doria-Rose et al., 2015; McCoy et

266 al., 2015). To see if VRC38.01 exhibits this phenomenon, we determined its maximum %

267 neutralization (Figure SC) against all 208 virus strains in Figure . To exclude weak neutralization

268 we only used data where IC50s were <1µg/ml. 80% and 72% were neutralized at >90% and

269 >95%, respectively. Similar patterns were observed for PG9 and PGT145. Sub-saturating

270 neutralization was somewhat more pronounced for CAP256.25 and was highly prevalent for

271 CH01, where only 5% of viruses were neutralized at a plateau >95%. In summary, like other

272 glycan V1V2 NAbs, VRC38.01 exhibits sub-saturating neutralization of some viruses.

273   An analysis of key V1V2 residues of our 208-member virus panel suggest that its side-

274 chain based binding mode (Figures 4H and 4I) may limit VRC38.01's breadth (Figure 5). Of the

275 148 VRC38.01-resistant strains, 137 related to variants at positions N130, N156 N160, K/R171

276 and Y173 (Figure 5A and 5B). Despite the VRC38.01 light chain contact with the poorly conserved

277 N133 glycan of the WITO.33 strain (Figure 3A and 3E), many N133-lacking viruses were

278 VRC38.01-sensitive. Variation at positions N160, K/R169 and K/R171 explained all 39 PG9-

279 resistant viruses (Figure S). N156, N160 and K/R171 variants explained most CH03-resistant

280 viruses, but 47 other resistant strains were unexplained (Figure S). Critical residues became

281 apparent when resistance mutations mapped onto mAb complexes (Figure 5C). VRC38.01

282 sensitivity was enhanced by K/R171 salt bridge with Glu97 of the heavy chain and by Y173

283 reorient N156 (Figure 5C). Conversely, the N130 glycan appeared to cause a light chain clash

284 (Figure 5C). PG9 sensitivity was enhanced by glycan binding and by electrostatic interactions

285 between Lys/Arg169 and Tys100g as well as Lys/Arg171 and Asp100i (Figure SC). CH03

286 sensitivity was also enhanced via glycan interactions and by a salt bridge formed between

287 Lys/Arg171 and Glu30 of the heavy chain (Figure SD).

288   Introducing the N130 glycan into 8 viruses that naturally lack this glycan reduced

289 VRC38.01 sensitivity of all virusesby >50-fold in 7 cases (Figure 5D), but had less on PG9 and

even less on PGT145, which neutralized a N130 glycan-bearing DU422.01 mutant more effectively (Figure 5D). These findings are consistent with the latter bnAbs targeting epitopes closer to the trimer vertex. Reciprocal removal of the N130 glycan increased VRC38.01 sensitivity of BaL.26 and 25711-2.4 by >40-fold and CAAN.A2 by >7-fold (Figure 5E). VRC38.01 achieved 47.5% neutralization of strain DU172.17 at 50 µg/ml, but did not neutralize the parent virus (Figure 5E). Again, the effect of removing glycan was less pronounced PG9 and PGT145, and in fact reduced PG9 sensitivity of the 25711-2.4 strain (Figure 5E). Autologous N90 donor viral sequences (Wu et al., 2012) exhibited key contacts for VRC38 but had a sequon at N130 (3) and were therefore VRC38.01-resistant. Upon N130D mutation, VRC38.01 reached 50% neutralization for 3 of viruses (Figure S7E). For the other 5 viruses tested, we detected increased VRC38.01 sensitivity that did not reach 50% at 100 µg/ml (Figure S).

Further mapping against strand A, B, and C mutants of the WITO.33 strain (4) revealed sequon-disrupting S158A, N160K, and T162A mutations in strand B sensitivity to all four V1V2 NAbs (4). N156A mutant was poor infection. Strand C mutations revealed K171 and Y173 critical for VRC38.01 but less so for other V1V2 NAbs. Analysis of BG505 and JR-CSF.JB strain mutants indicated similar VRC38.01 contacts (4). However, unlike WITO.33, Y173A mutation rendered these strains completely PGT145-resistant and K171A mutation rendered JR-CSF but not BG505 resistant to CH01. Overall, complete disruption by K171 or Y173 confirm strand C dependency. Together, sequence analysis of sensitive and resistant viruses (Figure 5) and mutation analysis (4) indicated VRC38 resistance largely explained by a N130 or by key residues at positions 171 and 173, and/or glycans at positions 156 and 160.

VRC38.01 olerates arge annose ranches

The glycosylation inhibitor kifunensine prevents trimming of high mannose glycans, resulting in largely uniform $Man_9GlcNAc_2$ structures. Pseudoviruses prepared with this inhibitor are resistant to V1V2 bnAbs (Doores and Burton, 2010), perhaps due to changes in apex folding and/or because of a loss of mAb contacts with glycan sialic acid termini in the absence of this inhibitor (Amin et al., 2013; Pancera et al., 2013). We found that 83% (PG9), 92% (CH01), 94% (PGT145) and 69% (CAP256.09) of kifunensine viruses were >4-fold resistant (S), but only 38% were resistant to VRC38.01. This further distinguishes VRC38.01 from other V1V2 mAbs and is in line with structural evidence suggesting that it can accommodate large mannose branches.

Ontogen VRC38 eutraliz

A phylogenetic tree of all 11 lineage members was generated, rooted by a common inferred ancestor (IA, Figure 6A). A variant with germline V-gene and J-gene reverted heavy (gH) and light (gL) chains, but maintaining the mature CDRH3, was unable to neutralize (Table S1). However, hybrids consisting of mixed mature and reverted heavy and light chain partners, gHL and HgL, neutralized 19 and 18 strains of a 208-member panel, respectively (). Fourteen strains were sensitive to both hybrids, while others were neutralized by only one hybrid, indicating that both chains contribute to neutralization. When both the gHL and HgL Abs were effective, HgL potency was generally stronger, suggesting that the mature heavy chain plays a dominant role (). Overall, this suggests that VRC38 can neutralize ~9% of heterologous strains with no maturation of the V-gene of one chain.

VRC38 heavy and light chain maturation was modeled in Figure 6 and Table S6, respectively. S31D mutation likely occurred early in the VRC38 heavy chain (Figure 6A and boxed starting at sequence I1 in Figure 6B) and forms a salt bridge with a 97% conserved basic residue at position 168 (Figure 6C). However, data above (Figure 1E) suggests that, unlike other V1V2 NAbs, this contact is not essential (Figure SB). Indeed, a D31S mutant modestly reduced neutralization of 11 of the 14 strains tested (Figure 6D). Two exceptions were BG1168.01 was more sensitive to the D31S mutant and ZA012.29 was insensitive to either the parent mAb o D31S mutant.

VRC38.01 share a branch with broad lineage members VRC38.07 and .06, to a S97E mutation acquired at intermediate I3 (Figure 6A-C), that forms a salt bridge with K171 of strand C (Figure 6C). E97S reversion reduced VRC38.01 neutralization to a larger degree than the D31S mutant. The two reversions together led to loss in neutralization, suggesting that both contacts contribute to VRC38.01 neutralizing activity (Figure 6D). VRC38.09, which is almost as broad as the three members of the VRC38.01 branch but almost 3-fold lower in potency, lacks the S97E mutation. However, a S97E mutant increased its potency agains 12 of the 14 strains (Figure 6E). Exception were uniquely VRC38.09-sensitive strain X1632.S2.B10 (Figure 1E) and the BG1168.01 strain. The behavior of the latter mirrored the effect of the reciprocal mutation on VRC38.01 (Figure 6D and E). Overall, VRC38.09's breadth developed independently from other clones and the sensitivity of some strains to this variant depend on different somatic mutations.

VRC38-like NAbs ay e revalent in HIV-nfected onors

To analyze VRC38-like bnAb prevalence, we used neutralization fingerprinting (Doria-Rose et al., 2017; Georgiev et al., 2013) to delineate bnAb specificities in a cohort of HIV-1 infected donors (Hraber et al., 2014). VRC38-like specificities (Figure 7A) were common,

357   appearing in ~20% of plasmas, PG9-like bnAbs. nalysis of the frequency of paired specificities
358   (Figure 7B) revealed that VRC38-like and PG9-like signals were frequently paired with 10E8-like
359   bnAbs - more frequently than 10E8-like pairings with other Abs. VRC38-like and PG9-like Abs
360   also paired with each other, albeit at a much lower frequency than with 10E8. Overall, VRC38-
361   like NAbs appear to be common in HIV-1 infection.

DISCUSSION

growing number of recovered bnAbs now cover most of the Envtrimer surface. Many have unusual features that set a high bar for vaccine design and justify continued efforts to uncover new specificities. The VRC38 lineage provides a previously unknown solution for V1V2 apex binding, with potential advantages. Abs with >24 AA CDRH3s result from uncommon recombination events and, due to their typically autoreactive nature, may not survive B cell tolerance checkpoints (Briney et al., 2012a; Briney et al., 2012b). In contrast, the length and charge of the VRC38 CDRH3 is within the normal repertoire range. The lack of CDRH3 tyrosine sulfation or prevalent anionic side chains further advance VRC38 as a tangible vaccine blueprint, although this possibility will require formal testing.

Our fingerprint analysis suggested that VRC38-like Abs may be as prevalent as PGT128-like or PG9-like Abs. We are now mapping donor sera with VRC38-like signals and probing their B cells. Given its common features, VRC38-like NAbs might have been expected to be even more . However, N130 glycan is found in >60% of circulating virusesthe single largest regulator of VRC38's breadth prevent VRC38-like bnAb development or facilitate escape. Accommodating glycan might therefore be a route to improved VRC38 breadth. We are now assessing additional donors and modifying B cell probe strategy. For example, in a variation of the strategy in Figure 1C, B cells could be gated for positive binding to bearing a N130 glycan knock in mutation. It may also be useful to interrogate the N90 donor for VRC38 signatures by NGS and to probe samples from other donors that exhibit VRC38 fingerprint signatures. In a vaccine setting, N130 clash could be addressed using priming immunogens bearing a N130 "glycan-hole" (Crooks et al., 2015) to promote VRC38-like NAb development and boosting with N130 glycan-containing immunogens to select for clones that can accommodate the glycan and thereby acquire greater breadth. Feasibility is supported by the observations that CD4bs-specific bnAb N6 evolved greater breadth by accommodating V5 loop glycans (Huang et al., 2016) and that the PGT121 family gained breadth by accommodating the N137 glycan (Garces et al., 2015).

Like other V1V2 NAbs (except PGT145 VH1-8), VRC38 VH3, the most common VH family in human B cell repertoires. Moreover, like other V1V2 bnAbs, VRC38 VH diverged <20% from its germline. This contrasts with the higher (~40%) divergence of CD4bs bnAbs, making the V1V2 an attractive vaccine target. Indeed, the marginal protective efficacy observed in the RV144 Thai vaccine trial was related to V1V2-specific Abs, albeit ones that were non-neutralizing and may therefore have exerted antiviral pressure by other mechanisms (Liao et al., 2013).

Some VRC38 binding requirements are akin to those of other V1V2 NAbs, including N156

396   and N160 glycan-dependen quaternary epitope preference and binding. In several other ways,
397   however, VRC38 is unique. First, it binds farther away from 3-fold trimer axis and both Fab arms
398   of VRC38.01 IgG bind to a single trimer. It also exhibits a different binding mode that depends
399   on CDRH1, CDRH3 and CDRL1 interactions with side-chains of strands A, B, and C rather than
400   main-chain interactions. This decreases the distance the CDRH3 must travel to penetrate the
401   glycan shield. Since these contacts are conserved, VRC38 exhibits moderate breadth. Second,
402   VRC38's CDRH3 exhibits a highly aromatic motif consisting of seven AAs (WFYHYYW), allowing
403   it to glycans N156 and N160. Its unusual kifunensine resistance, along with structural data,
404   suggest that it engages these glycans predominantly via their invariant stems. Thus, although
405   glycan form variations may not affect VRC38, other V1V2 NAbs that depend for example on sialic
406   acid contacts could be more prone to viral escape due to natural glycovariation (Amin et al., 2013;
407   Pancera et al., 2013). Alternatively, the larger $Man_9GlcNAc_2$ structures promoted by kifunensine
408   may distort packing at the trimer apex, perhaps impacting inter-protomer binding by some V1V2
409   bnAbs (Julien et al., 2013), but not VRC38 because it binds largely within one protomer. Unlike
410   other V1V2 bnAbs (except CH01-CH04), VRC38.01's three HCDR3 tyrosines are not sulfated
411   and lack anionic character. Specifically, YYDbinding motifs (with both tyrosines carrying charged
412   sulfates) of PG9 and VRC26 are absent. Thus, unlike other V1V2 bnAbs, VRC38.01 binding is
413   not heavily dependent on electrostatic interactions.
414   Although VRC38.01 depends on residues K171 and Y173 of strand C, its partial K168-
415   independence uniquely allows it to neutralize strains that are insensitive to other V2 mAbs. While
416   K171 makes a clear electrostatic interaction with VRC38.01, Y173 may indirectly contribute by
417   reorienting the N156 glycan. The lack of sensitivity of clade D viruses to VRC38.01 is explained
418   by the fact that they typically lack the critical K/R171 and Y173 contacts. The higher clade A
419   breadth of VRC38.01 was surprising, considering the donor's infection with a clade B virus and
420   North America origin (Wu et al., 2012). However, clade A preference is also true for other V1V2
421   bnAbs, albeit those derived from African origin. However, although VRC38.01 breadth against
422   clade A and clade A hybrid viruses was relatively high, it did not exceed that against the infecting
423   clade B.
424   The cells from which the VRC38 lineage was cloned were sampled many years after
425   infection, making it difficult to trace . A common unmutated ancestor and intermediates were
426   inferred from the 11 Ab sequences derived from the single time point. eutralization by the reverted
427   VRC38.01 Ab and hybrid Abs with mature chains indicated both chains contribute to
428   neutralization, the heavy chain making a greater contribution. Two affinity matured residues on
429   the heavy chain (S31D and S97E) that form salt bridges present in the broadest clones. The

430   neutralizing activity of the gHL variant suggests that while these interactions are not critical, they
431   increase breadth and potency.

432   VRC38.09, neutralized a virus that was insensitive to the VRC38.01 branch. Another virus
433   was uniquely neutralized by the VRC38.14 variant heavy chain when paired with VRC38.01 light
434   chain. Combining the number of viruses neutralized by all variants brought the VRC38 lineage
435   breadth to 48%. It is unclear if a pathway could be found to neutralize these strains (and perhaps
436   more) by a single VRC38 clone, especially since we only have access to one timepoint in this
437   donor and thus how the lineage might have evolved.

438   Our success in recovering VRC38 may in part be due to extensive B cell probe
439   development. The broadest lineage member, VRC38.01, was identified using trimer VLPs as
440   probes and others were then isolated using SOSIP trimers. Thus, VLPs may complement the
441   growing list of B cell probes (Doria-Rose et al., 2015; Kong et al., 2016; Sok et al., 2014; van Gils
442   et al., 2016). The high frequency of NAb clones in the VLP sort (2 out of 92; 2.2%) was consistent
443   with specific staining. Nevertheless, certain suboptimal aspects of VLPs could be improved.
444   Trimer VLPs imparted smeared staining in which VRC38.01 and VRC38.02 were relatively dim
445   on the GFP axis, as opposed to a clearly separated positive population commonly seen with
446   soluble SOSIP trimers. We are currently investigating alternative conjugation technologies
447   (Mengistu et al., 2015) to tag VLPs with brighter fluorophores to enhance positive stain separation
448   from background. Our observation that VRC38.01 contributes only partially to N90 serum breadth
449   suggests other bnAbs exist in this donor that we are now trying to recover.

450   In summary, a VLP probe recovered a VRC38 NAb lineage targeting the trimer apex
451   without protruding binding loops by a previously unrecognized mechanism involving side-chain to
452   side-chain interactions that reduced the distance an Ab loop must traverse the glycan shield. The
453   genetic, functional and structural analysis of this lineage, together with serological fingerprint
454   analysis, suggest that similar NAbs may be relatively common during HIV-1 infection and may
455   thus be amenable to vaccine elicitation.

AUTHOR CONTRIBUTIONS

Conceptualization, EMC, JG, NAR, PDK, JRM, and JMB; Methodology, EMC, JG, ETC, TT, NDR, TBK, ABW, ISG, JMB, JRM, and JMB; Software, TBK and ISG; Validation, RTB and MKL; Investigation, EMC, JG, NAR, ETC, KO, TT, JL, RN, GO, DRA, MA, AKB, YF, SO, CO, and TBK; Resources, ETC, TT, XC, AD, MGJ, MP, MC, TJH, and RTW; Writing – Original Draft, EMC, JG, and JMB; Writing – Review & Editing, EMC, JG, PDK, JRM, and JMB; Visualization, EMC, JG, GO, ISG, and JMB; Supervision, ABW, ISG, PDK, JRM, and JMB; Project Administration, EMC, JG, PDK, JRM, and JMB; Funding, PDK, JRM, and JMB.

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FIGURE LEGENDS

477

478   **Figure 1. Trimer VLP probes identify a NAb lineage of moderate CDRH3 length from an HIV-**

479   **1-infected donor. A)** Overview of VLP-based NAb isolation. Negatively selected CD19+ B cells

480   labeled with GFP-trimer VLPs and RFP-bald VLPs, then singly indexed sorted for GFP$^+$RFP$^-$ B

481   cells. Immunoglobulin genes were RT-PCR amplified. Desirable sequences were cloned,

482   expressed and characterized. **B)** GFP$^+$RFP$^-$IgG$^+$ B cells (green dots) were selected. Dark green

483   dots indicate VRC38.01 and .02. **C)** PE$^+$APC$^-$IgG$^+$ B cells (pink dots) were selected from SOSIP-

484   gp140F stains. Red dots indicate VRC38.03-11. **D)** VRC38 lineage heavy and light chain

485   sequences were aligned to their closest V-gene and J-gene germline genes. Clones isolated

486   using VLPs or SOSIP trimers were colored in green and red, respectively. *Mutation frequencies

487   refer to percent nucleotide sequence divergence from germline V-gene sequences. **E)** VRC38.01-

488   11 neutralization of a multi-clade virus panel. GMTs of sensitive viruses (IC50<50µg/ml) and

489   percent of viruses neutralized at IC50<50µg/ml (breadth) are shown. Although VRC38.05

490   neutralized only one virus, for comparison, this value was included in the "mean IC50" row.

491   Neutralization assays were repeated 2 times. See also Figures S1-S.

492

493   **Figure 2. VRC38.01 a quaternary epitope at the V1V2 apex. A)** Excess VRC38.01 was used

494   to compete with graded concentrations of biotinylated or strepII-tagged mAbs (VRC01 and

495   35O22) for binding to JR-FL SOS E168K or BG505 (for CAP256.09) trimer VLPs by ELISA. **B)**

496   Binding to SOSIP.664 gp140 trimers and monomeric gp120 of strains Q23.17 and DU156.12 by

497   ELISA. **C)** Binding to various monomeric gp120s by ELISA. **D)** Negative-stain EM of VRC38.01-

498   SOSIP.664 complexes. Representative reference-free 2D class averages of known V1V2 bnAbs

499   (top panel) and VRC38.01 IgG or Fab (bottom panel) complexed with BG505 SOSIP.664. In the

500   middle panel, a ligand-free SOSIP model (low-pass filtered EM map created from x-ray

501   coordinates of PDB 4ZMJ) shows various V1V2 bnAb epitopes. PG9 and CAP256.09 Fabs are

502   shown by EM volumes and PGDM1400 and VRC38.01 footprints are indicated. For VRC38.01

503   Fab and IgG, proportions of complexes showing 1 Fab or 2 Fabs binding the trimer are shown.

504   ELISA assays were repeated 2 times. See also Figures S2, S.

505

506   **Figure 3. V1V2 scaffold complexes reveal VRC38.01-glycan contacts and "off center" apex**

507   **binding. A)** A VRC38.01 V1V2-1FD6 scaffold co-crystal was solved at 3.5 Å. The heavy and light

508   chains are shown in yellow and blue. The V1V2 (residues 126-196) and 1FD6 scaffold are shown

509   in magenta and grey, respectively. Glycan mannoses are shown in green, and *N-*

510 acetylglucosamine moieties are shown in blue. **B)** CDRs are highlighted for the bound Fab, which
511 did not vary from the unbound Fab (). **C)** The V1V2s of the WITO.33 scaffold (magenta) and
512 BG505 SOSIP.664 trimer (pink, PDBID 4TVP) were aligned with an RMSD of 1.1 Å in the ß-
513 sheets. **D)** The highly aromatic CDRH3 loop of VRC38.01 is enveloped by N156 and N160
514 glycans. Underlined CDRH3 AAs are shown in stick representation. **E)** A quantitative analysis of
515 the mAb-glycan contacts is shown, with buried surface area of *N*-acetylglucosamine (blue
516 squares) and mannose (green circles) residues indicated. **F)** Alignment of the VRC38.01-V1V2
517 complex with the BG505 SOSIP trimer structure (4TVP) via the V1V2 domain. See also Figure.
518
519 **Figure 4. VRC38.01's non-protuding HCDR3 makes side chain-side chain contacts at the**
520 **V2 apex. A)** Key features of prototype V1V2 mAbs, including their source, neutralization breadth
521 (see Figure S6), germline VH gene use and divergence (nucleotide), CDRH3 length, sequence
522 and net charge. Negatively charged residues are shown in red and sulfated tyrosines are
523 underlined. Each NAb is color coded throughout. **B)** V1V2 mAb CDRH3 lengths (indicated by
524 colored arrows) compared to the naïve Ab repertoire distribution (Shi et al., 2014). **C)** Net V1V2
525 NAb CDRH3 charge compared to the naïve Ab repertoire. **D)** V1V2 mAb germline divergence
526 compared to the naïve Ab repertoire. **E)** Alignment of the 5 mAb structures via their framework
527 regions. **F)** Sulfated tyrosines are shown as sticks and the electrostatic surface representation
528 depicts charge. **G)** V1V2 mAb CDRH3 loops complexed with the V1V2 (magenta) and associated
529 glycans (surface). **H)** VRC38.01, PG9, and CH03 interactions with V1V2 strand C. VRC38.01
530 makes side-chain hydrogen bond contacts with K168 and K171 of strand C, N156 and S158 of
531 strand B, and N133 and T135 of strand A. PG9 and CH03 make extensive main-chain parallel
532 strand-strand interactions only with strand C. Side chain PG9 and CH03 contacts are shown in
533 Figure S9B. **I)** Summary of V1V2 mAb contacts. Aligned V1V2 sequences are shown with their
534 VRC38.01 sensitivities. VRC38.01, CH03 and PG9 contacts are shown as closed circles (side
535 chain contacts), open circles (main chain contacts) and starred circles (both side chain and main
536 chain contacts). Strands A to D, the N133, N156 and N160 glycans and the site of an engineered
537 *N*-linked glycosylation insert between strands B and C (residues 165 and 166) placed at the
538 approximate location of the N160 glycan of the neighboring protomer. See also Figure S5.
539
540 **Figure 5. Breadth and residue-level requirements of VRC38.01 sensitivity.** Prototype V1V2
541 mAbs were color coded as in Figure 4. **A)** Sequences of VRC38.01-sensitive (IC50<50μg/ml) and
542 -resistant (IC50>50μg/ml) strains of our 208-member virus panel (Table S1) were rendered as
543 logo plots, the height of each residue corresponding to its frequency. Residues and sequons (gly)

544 linked with sensitivity (green) or resistance (red) with % conservation are shown. **B)** VRC38.01
545 resistance mutations in descending order of prevalence, with red bars indicating the number of
546 viruses containing each resistance mutation. Vertical red bar overlaps indicate strains with
547 multiple resistance mutations. Gray bars indicate the number of viruses with unaccounted
548 resistance mechanisms. **C)** Structural analysis of VRC38.01 resistance mutations. A common
549 glycan at residue 130 clashes with the VRC38.01 light chain (left). A favorable electrostatic
550 interaction is formed between Glu97 of the heavy chain and a positive residue at position 171 on
551 gp120 (center). Tyr173 appears to favorably orient the N156 glycan (right). **D)** Effects of N130
552 glycan knockin (KI) mutations on the V1V2 NAb sensitivity of viruses that naturally lack the N130
553 glycan. IC50 ratios against the parent and each KI virus are indicated. Ratios >1 indicate
554 increased sensitivity. Conversely, ratios of <1 indicate reduced sensitivity. **E)** Effects of N130
555 glycan removal (KO) mutations on the V1V2 NAb sensitivity of viruses that naturally bear this
556 glycan. IC50 ratios are shown as in (D). *Maximum neutralization (50μg/ml) of the DU172.17
557 mutant by VRC38.01 reached 47.8%, as compared to 0% for WT. Neutralization assays were
558 repeated 2 times. See also Figures S.

559

560 **Figure 6. Structural maturation of the VRC38 lineage sequence. A)** VRC38 phylogenetic tree
561 branches were color-coded by their breadth against 31-member virus panel (Figure 1E).
562 Computationally inferred intermediates are shown at nodes, with those leading to mature
563 VRC38.01 shown in blue. S31D and S97E mutations are shown as they first appear. **B)** Heavy
564 chain intermediate (upper set) and mature (lower set) sequences. In the upper set, intermediates
565 leading to VRC38.01 are shown in blue. In the lower set, the 11 mature heavy chains are shown
566 in descending order of breadth against the 31-member virus panel and are color-coded as in
567 panel A. D31 and E97 contacts are boxed. **C)** Structures of germline-reverted (gHgL, grey), I1
568 and I3 intermediates, and mature Ab complexed with WITO V1V2 (magenta). VRC38.01 heavy
569 (yellow) and light (blue) chain V1V2 contacts are colored and shown in stick representation as
570 they appear during maturation. **D)** Effects of reverting D31 and E97 residues to VRC38.01 inferred
571 ancestor residues (S31 and S97) on mature VRC38.01 neutralization activity. Mutations, alone or
572 in combination, were introduced into the VRC38.01 heavy chain, and co-expressed with the
573 mature VRC38.01 light chain, then assessed for neutralization activity. Ratios of IC50s of WT
574 VRC38.01 and mutant Abs are shown. Data are excluded for viruses in which both the WT and
575 mutant Abs yielded IC50s>50μg/ml. **E)** Effects of mutating VRC38.09 S97 to E97. The heavy
576 chain mutant was co-expressed with the mature VRC38.09 light chain and assessed for

577 neutralization. Ratios of IC50s of WT VRC38.09 and of the S97E mutant Ab are shown.
578 Neutralization assays were repeated 2 times. See also Table S6.
579
580 **Figure 7. Fingerprint analysis suggests VRC38-like specificities are common in HIV-**
581 **infections. A)** Predicted frequency (percent of plasma) of different bnAb specificities. Bars were
582 colored per Ab specificity, with bar height corresponding to frequency. **B)** Frequency of paired
583 bnAb specificities in samples with predicted multiple specificities. Circle diameter corresponds to
584 bnAb specificity frequencies. Edge widths correspond to the frequencies of specificity pairs.
585
586 **STAR ★ METHODS**
587
588 **CONTACT FOR REAGENT AND RESOURCE SHARING**
589 Further information and requests for resources and reagents should be directed to and will be
590 fulfilled by the Lead Contact, James M. Binley (jbinley@SDBRI.org).
591
592 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
593
594 <u>Human Subjects</u>
595 Archived peripheral blood mononuclear cells (PBMCs) and sera derived from HIV-1
596 infected donor N90 were used as a source of B cells. This donor signed informed consent and
597 participated in NIAID protocols at the National Institutes of Health, Bethesda, MD **(Doria-Rose et**
598 **al., 2010; Georgiev et al., 2013; Migueles et al., 2002)**. At the time point studied, this donor was
599 an antiretroviral therapy-naïve slow progressor who had been diagnosed 23 years prior and
600 exhibited broad serum neutralization, with viral load of 8,216 copies/ml, and 912 CD4$^+$ T-cells/µl
601 **(Wu et al., 2012)**. A cohort of serum samples from donors infected with various HIV-1 clades
602 assembled from various sources, including the Center for the AIDS Programme of Research in
603 South Africa (CAPRISA), the Center for HIV/AIDS Vaccine Immunology (CHAVI) and others, as
604 previously published **(Hraber et al., 2014)** was used for fingerprinting analysis. Serum from
605 chronically infected donor 1686 was previously shown to broadly neutralize via epitope(s)
606 overlapping the CD4 binding site **(Binley et al., 2008)**. PBMCs with no donor identifiers were also
607 obtained from seronegative subjects for bait optimization.
608
609 <u>Cell Lines</u>

610   Human embryonic kidney (HEK)-derived 293T, HEK 293S N-
611   acetylglucosaminyltransferase I-negative (GnTI-), and HeLa-derived TZM-bl cells were
612   maintained in complete Dulbecco's Modified Eagle Medium (herein referred to as cDMEM)
613   containing high-glucose Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher, Waltham,
614   MA), 1X Penicillin-Streptomycin (Pen Strep, Thermo Fisher) and 10% fetal bovine serum (FBS,
615   Gemini Bio Products, West Sacramento, CA) at 37°C/5% CO_2. Canine thymus-derived
616   CF2Th.CD4.CCR5 cells were maintained in DMEM containing 1X Pen Strep, L-glutamine, 500
617   □g/ml G418, 150 □g/ml Hygromycin, and 10% FBS at 37°C/5% CO_2. FreeStyle 293F and
618   Expi293F cells (both Thermo Fisher) were maintained in Freestyle 293 Expression Medium and
619   Expi293 Expression Medium, respectively, at 37°C/10% CO_2 with shaking at 120 RPM. Human
620   Burkitt's B cell lymphoma-derived Ramos cells were maintained in either DMEM containing 15%
621   FBS and 1X Pen Strep or Roswell Park Memorial Institute 1640 medium (RPMI 1640, Thermo-
622   Fisher) containing 10% FBS and 1X Pen Strep at 37°C/5% CO_2. In some cases, Ramos cells
623   were engineered to stably express mature B cell receptors (BCR) IgM versions of VRC01,
624   PGT145, and PGT128.
625
626   **METHOD DETAILS**
627
628   Anti-HIV-1 Env Monoclonal Abs
629   MAbs were obtained from the NIH AIDS Reagent Repository or from their originators.
630   Further information can be found in the HIV Molecular Immunology Database
631   (http://www.hiv.lanl.gov/) and in ref **(Burton and Hangartner, 2016)**. MAbs included the following
632   (originators given in parentheses): 2G12 (H. Katinger), directed to a glycan cluster on gp120 ;
633   39F and CO11 (J. Robinson), directed to the gp120 V3 loop; b12 (D. Burton), VRC01, VRC07-
634   G54W, VRC13 (J. Mascola) and HJ16 (A. Lanzavecchia) directed to epitopes that overlap the
635   CD4bs; PGT121, PGT125 and PGT128 (D. Burton) directed to epitopes involving the base of the
636   V3 loop of gp120 and the N332 glycan; PG9, PG16, PGT145, PGDM1400 (D. Burton), CH01 and
637   CH03 (B. Haynes) and CAP256.01-33 (J. Mascola), directed to quaternary, glycan-dependent
638   V1V2 loop-directed epitopes; PGT151 (D. Burton), 35O22 (M. Connors), and 8ANC195 (M.
639   Nussenzweig), directed to the gp120/gp41 interface; 10E8 (M. Connors), 2F5 and 4E10 (H.
640   Katinger), directed to the gp41 membrane-proximal ectodomain region (MPER)**.**
641
642   Plasmids and Mutagenesis

643   Plasmid pCAGGS was used to express JR-FL SOS E168K gp160ΔCT **(Moore et al.,**
644   **2006),** featuring a 3 amino acid gp41 cytoplasmic tail, "SOS" mutations (A501C and T506C) to
645   introduce a disulfide bond between gp120 and gp41 **(Binley et al., 2000)** and an E168K mutation
646   to "knock in" V1V2 bnAb epitopes **(Doores and Burton, 2010; Tong et al., 2012)**. Various Env
647   expression plasmids were used to pseudotype viruses for neutralization assays and to express
648   soluble gp140 glycoproteins, including uncleaved (UNC) YU2 gp140 foldon (gp140F) and
649   BG505.DS.T332N.SOSIP.664 gp140 trimers (termed BG505 SOSIP trimers elsewhere for
650   brevity) that feature the stabilizing 201C-433C disulfide (DS) mutation **(Kwon et al., 2015).** Some
651   clones featured a C-terminal Avi tag (GLNDIFEAQKIEWHE) to enable biotinylation and
652   fluororphore-streptavidin complexing **(Doria-Rose et al., 2015).** A codon optimized WITO strain
653   V1V2 1FD6 scaffold was designed based on a previous construct **(McLellan et al., 2011)** and
654   features an artificial N-terminal secretion signal (MRPTWAWWLFLVLLLALWAPARG), a C-
655   terminal HRV3C cleavage site (GLEVLFQGP) followed by an 8-His tag and an engineered N-
656   linked glycosylation site between strands B and C (residues 165 and 166) at the approximate
657   location of the N160 glycan from the neighboring protomer. Env-deficient sub-genomic plasmids
658   pNL4-3.Luc.R-E- and pSG3ΔEnv were described previously **(Li et al., 2005).** Plasmids pMV-2024
659   and pMV-ERV express full-length SIVmac251 (BK28) and MLV Gag, respectively. Plasmid pMV-
660   0932 that expresses HIV-1 Rev was co-expressed whenever VLPs were produced using SIV or
661   MLV Gag **(Crooks et al., 2015).** Plasmids pGag-GFP and pGag-RFP express full-length HIV-1
662   p55 Gag fused to eGFP and mCherry fluorphores, respectively, that for convenience are referred
663   to as GFP and RFP throughout this study **(Gomez and Hope, 2006).** MAb heavy and light chain
664   genes (in many cases codon optimized) were inserted into pVRC8400 to express IgG in Freestyle
665   293F or Expi293F cells, followed by protein A purification. In some cases, heavy chain plasmids
666   were modified to add a C-terminal streptactin II tag
667   (GGPGSAWSHPQFEKGGGSGGGSGGSAWSHPQFEK), a HRV3C recognition site
668   (LEVLFQ/GP) inserted after K235 of the heavy chain and/or M428L/N434S mutations (indicated
669   by a "-LS" suffix) **(Zalevsky et al., 2010),** to improve neonatal Fc receptor binding and *in vivo* half
670   life.
671
672   <u>Ramos-IgM Cell Line Production</u>

673   Ramos cells were engineered to stably express mature B cell receptors (BCR) IgM
674   versions of VRC01, PGT145, and PGT128.by lentiviral transduction of the cells using FEEKW-
675   vectored light chain and IgM heavy chain-expressing lentiviruses. BCR-positive cells were
676   identified by staining with both PE-conjugated mouse anti-human-kappa/lambda (BD

23

677 Biosciences, San Jose, CA) and anti-human-IgM (Biolegend, San Diego, CA) by flow cytometry

678 and sorted using a FACS Aria (BD Biosciences). Cells were amplified, assessed for BCR

679 expression as before, and resorted for highest-staining cells if needed.

680

681 <u>VLP Production</u>

682   VLPs were produced by co-transfecting 293T or Freestyle 293F cells with plasmids

683 expressing Env, Gag, and Rev **(Crooks et al., 2015)**. Fluorescent VLPs were produced by also

684 cotransfecting plasmids pGag-GFP or pGag-RFP. Two days later, supernatants were collected,

685 filtered, precleared by centrifugation at 450Xg for 5 minutes, and concentrated either by tangential

686 flow filtration or by centrifugation at 50,000Xg in a Sorvall SS34 rotor. "Trimer-VLPs" were made

687 by digesting with 1□l each of 1mg/ml proteinase K, subtilisin, and trypsin, and 1□l of 2mg/ml

688 chymotrypsin **(Crooks et al., 2011; Tong et al., 2012).** pNL4-3.Luc.R-E- based fluorescent VLPs

689 were inactivated using 1mM aldrithiol (AT-2) before use as baits **(Crooks et al., 2015)**. RFP-

690 labeled "Bald-VLPs" were produced by co-expressing pMV-2024 (expressing SIV Gag) and

691 pGag-RFP plasmids.

692

693 <u>Soluble Env Protein Production</u>

694   Trimeric Env constructs were expressed in various types of 293 cells and then purified by

695 previously published methods **(Bhiman et al., 2015; Doria-Rose et al., 2015; Stewart-Jones**

696 **et al., 2016)**. For BG505.T332N.SOSIP proteins used for flow cytometric cell sorting, Avi-tagged

697 proteins were biotinylated using the BirA Biotin-Protein Ligase Bulk Reaction Kit (Avidity, LLC,

698 Aurora, CO) under the following conditions and reagents from the kit: 25□l of 10X Biomix A, 25□l

699 of 10X Biomix B, 20□g of BirA protein, and 180□l of Avi-tagged SOSIP protein, incubated for 5

700 hours at 30°C with constant agitation. The mixture was buffer exchanged into SOSIP buffer

701 containing 5mM HEPES, 150mM NaCl, and 0.02% NaN_3 in 30,000 MWCO Amicon Ultra-15

702 centrifugal filter units (EMD Millipore) over 5 rounds of spinning at 3000Xg, 4°C. Biotinylated

703 SOSIP was then conjugated with streptavidin, R-phycoerythrin (SA-PE, Thermo Fisher) at a 1.17

704 SA-PE:SOSIP weight ratio (determined empirically). Briefly, biotinylated SOSIP was mixed with

705 1/5 the final volume of SA-PE and incubated for 20 minutes at 4°C with gentle rotation.

706 Subsequent 1/5 increments of SA-PE were then added with 20-minute incubations in between

707 each addition. APC-conjugated YU2 gp140 foldon (gp140F) protein was biotinylated and

708 conjugated to SA-allophycocyanin (SA-APC, Thermo Fisher) in the same manner at a 1.8

709 gp140F:SA-APC weight ratio (determined empirically)

710   V1V2 regions of various strains (residues 126-196, HXB2 numbering) were scaffolded

711 onto a core variant of protein G (PDB 1FD6) and were produced in HEK 293S GnTI- cells and

712 purified over Ni-NTA superflow resin (Qiagen, Hilden, Germany), then digested with HRV3C

713 (Novagen, Darmstadt, Germany) and purified over a 16/60 S200 size exclusion column **(McLellan**

714 **et al., 2011; Ross et al., 2001)**.

715

716 <u>HIV-1 Env-pseudotyped Virus Production</u>

717   Pseudoviruses for use in TZM-bl neutralization assays were produced in 293T cells by

718 cotransfection of a pSG3ΔEnv backbone plasmid and a full HIV-1 Env gp160-encoding plasmid

719 (Li et al., 2005). Briefly, $2X10^6$ cells in 20ml cDMEM were seeded in T75 flasks the day prior to

720 cotransfection. For transfection, 40□l of FuGene 6 reagent (Promega, Fitchburg, WI) was diluted

721 into 800□l of room-temperature Opti-MEM I reduced serum medium (Thermo Fisher), followed

722 by addition of 10□g of pSG3ΔEnv backbone plasmid. 3.3□g of HIV Env plasmid was then added

723 to the mixture, mixed, and incubated for 30 minutes at room temperature. Transfection mixture

724 was then added to media of previously seeded 293T cells in the T75 flask and then distributed

725 evenly on cells. The following day, media was replaced with 20ml fresh cDMEM. Virus was

726 harvested the following day by filtering cell supernatants with 0.45□m Steriflip units (EMD

727 Millipore, Darmstadt, Germany) and aliquotted.

728   Pseudoviruses for use in CF2 cell assays were produced in 293T cells by cotransfecting

729 pNL4.3.Luc.R-E backbone plasmid and Env-expressing pCAGGS plasmid using

730 polyethylenimine (PEI) Max (Polysciences, Warrington, PA). Briefly, $5X10^6$ cells were seeded on

731 a 15cm tissue culture dish one day prior to transfection. On the day of transfection, 10□g of

732 pNL4.3.Luc.R-E and 10□g of Env plasmid were mixed in 2ml of serum-free DMEM. 40□l of

733 1mg/ml PEI Max (DNA:PEI ratio 1:2) was added and incubated at room temperature for 30

734 minutes. 11ml of cDMEM was then added to the transfection mixture. 293T cells were washed

735 with PBS, and transfection mixture was added to the cells and distributed evenly. Following a 2-

736 hour incubation at 37°C, culture dishes were washed with PBS, and 25ml of cDMEM was added

737 to the cells for a 48-hour incubation at 37°C. Following incubation, virus was harvested by

738 pelleting cell culture supernatant at 450Xg and filtering supernatant through a 0.8□m filter.

739   For pseudoviruses containing point mutations, mutations were introduced into full HIV-1

740 Env-encoding plasmids using the QuickChange II Site-Directed Mutagenesis Kit (Agilent

741 Technologies, Santa Clara, CA) according to the manufacturer's directions. For pseudoviruses

742 produced under conditions containing kifunensine, 25□M kifunensine was added at the time of

743 plasmid cotransfection and replenished during the media change.

25

744

B Cell Sorting of Env Trimer-specific Clones

Frozen PBMCs (~5X10^7 cells) were thawed in cDMEM containing 50 U/ml of benzonase (Novagen). For flow cytometric sorting, B cells were first enriched by negative magnetic bead selection using the Human B Cell Isolation Kit II (Miltenyi Biotec, San Diego, CA), then washed in PBS, resuspended in LIVE/DEAD® Fixable Violet Dead Cell Stain (Thermo Fisher) and incubated for 30 minutes at 4°C. Cells were washed once in PBS and multicolor staining was performed using a panel of fluorophore-labeled mAbs directed to CD3 (APC-Cy7, clone SK7), CD8 (Brilliant Violet 711, clone RPA-T8), CD14 (Brilliant Violet 605, clone M5E2), CD19 (PE-Cy7, clone HIB19) and human IgG (fluorescein isothiocyanate [FITC] or Alexa Fluor 680, both clone G18-145). All mAbs were obtained from BD Biosciences except for those directed to CD8 and CD14 (Biolegend) and the anti-IgG-Alexa 680 Ab (custom-conjugated at the Vaccine Research Center, National Institutes of Health, Bethesda, MD). Cells were also stained with fluorophore-tagged VLPs, BG505 SOSIP trimers or YU2 gp140F. All staining was performed at 4°C for 30 minutes, followed by two PBS/10% FBS washes and resuspension in PBS. Cells labeled as CD3$^-$/CD8$^-$/CD14$^-$/CD19$^+$/IgG$^+$/GFP$^+$/RFP$^-$ memory B cells were gated for VLP stains, or as CD3$^-$/CD8$^-$/CD14$^-$/CD19$^+$/IgG$^+$/BG505 SOSIP trimer$^+$/YU2 gp140F$^-$ for BG505 SOSIP trimer stains. Desirable cells were singly index-sorted on a FACS Aria sorter (BD Biosciences) into 96-well PCR plates (Denville Scientific, Holliston, MA) containing 20□l/well lysis buffer consisting of 1U/□l RNAse OUT (Thermo Fisher), 0.3125% Igepal CA-630, 1X SuperScript III First-Strand Buffer and 6.25 mM dithiothreitol (DTT) provided with the Superscript III Reverse Transcriptase kit (Thermo Fisher). Data was collected using FACSDiva software (BD Biosciences). Indexed sort data was analyzed using FlowJo software (FlowJo, LLC, Ashland, OR).

Single Cell RT-PCR of Ig Genes

RT-PCR amplification of IgG heavy and light chain genes was performed as described previously **(Doria-Rose et al., 2015)**. 96-well plates containing single sorted cells were initially frozen at -80°C and thawed to maximize liberation of cellular mRNA. Total mRNA in each well of the 96-well plates was reverse transcribed using 200U/well Superscript III reverse transcriptase (Thermo Fisher), 2□l of 10mM dNTP mix (Bioline, Taunton, MA) and 3□l of 150 ng/ul random hexamers (Gene Link, Hawthorne, NY) under the following cycling parameters: 42°C 10 min, 25°C 10 min, 50°C 60 min, 94°C 5 min, and 4°C hold.

First strand cDNA was amplified in a 2-step multiplex nested PCR in which the first step used either mixed γ-, λ-, or κ-chain-specific primers (listed in **(Doria-Rose et al., 2015)**. 50□l □-

778 chain amplification reactions were performed using the Qiagen HotStarTaq Plus DNA Polymerase

779 Kit as follows (all reagents from HotStarTaq Plus kit unless otherwise noted): 1X Qiagen PCR

780 Buffer, 200□M dNTP mix (Bioline), 500□M MgCl$_2$, 1□l of 50□M 1st round forward primer mix (5'L-

781 Vk mix), 1□l of 25□M 1st round reverse primer (3'Ck 543), 2U HotStarTaq Plus, and 5□l of first-

782 strand cDNA, under the following cycling parameters: 95°C 5 min; 50 cycles of 95°C 30 sec, 58°C

783 30 sec, 72°C 1min; 72°C 7 min; and 4°C hold. 40□l □- chain and □-chain amplification reactions

784 were performed similarly as follows: 1X Qiagen PCR Buffer, 125□M dNTP mix (Bioline), 1.4mM

785 MgCl$_2$, 0.2□l of 50□M 1st round forward primer mix (G1, G2, or G3 mix for □- chain and 5'L-VL-

786 RL mix for □-chain), 0.2□l of 25□M 1st round reverse primer (3'CgCH1 for □- chain and 3'Cl for

787 □- chain), 2U HotStarTaq Plus, and 3□l of first-strand cDNA. □-chains were amplified under the

788 following cycling parameters: 94°C 5 min; 50 cycles of 94°C 30 sec, x^{o}C 30 sec, 72°C 55 sec;

789 72°C 10 min; and 4°C hold, where x = 54°C for G1 mix, 48°C for G2 mix, and 52°C for G3 mix.

790 □-chains were amplified under the following cycling parameters: 95°C 5 min; 50 cycles of 95°C

791 30 sec, 50°C 30 sec, 72°C 1min; 72°C 7 min; and 4°C hold.

792   The second multiplex PCR step used mixed chain-specific primers that were

793 complementary to regions slightly upstream and downstream from the first step forward and

794 reverse primers, respectively. 50□l □-, □- and □- chain amplification reactions were performed

795 using the Qiagen HotStarTaq Plus DNA Polymerase Kit as follows (all reagents from HotStarTaq

796 Plus kit unless otherwise noted): 1X CoralLoad PCR Buffer, 200□M dNTP mix (Bioline), 1X Q

797 Solution, 1□l of 50□M 2nd round forward primer mix (5'L-VH mix + 5xwl-VH mix for □- chain, 5'L-

798 Vk-MS mix for □- chain, and 5'L Vl mix for □- chain), 1□l of 25□M 2nd round reverse primer

799 (3'IgGint for □- chain, 3'Ck 494 for □- chain, and 3'XhoI Cl for □- chain), 2U HotStarTaq Plus,

800 and 3.5□l 1st round PCR product.   □-chains were amplified under the following cycling

801 parameters: 95°C 5 min; 50 cycles of 95°C 30 sec, 58°C 30 sec, 72°C 1min; 72°C 7 min; and 4°C

802 hold.   □-chains were amplified under the following cycling parameters: 95°C 5 min; 50 cycles of

803 95°C 30 sec, 52°C 30 sec, 72°C 1min; 72°C 7 min; and 4°C hold. □-chains were amplified under

804 the following cycling parameters: 95°C 5 min; 50 cycles of 95°C 30 sec, 60°C 30 sec, 72°C 1min;

805 72°C 7 min; and 4°C hold.

806   10□l of each second round PCR product was loaded into 1% ethidium bromide-stained

807 pre-cast gels (Embi Tec, San Diego, CA) and run at 120 volts. Gels were visualized under

808 ultraviolet light, and second round PCR products from wells where PCR-amplified bands were

809 obtained were selected for sequencing by ACGT, Inc (Wheeling, IL). Alignments of sequences

810 to germline V-, (D-), and J-genes and junctional analyses were performed using IMGT/V-QUEST

811 (www.imgt.org). Clones of interest were identified by several criteria, including substantial

812 germline mutations (>10%) indicating a history of affinity selection, long CDRH3 loops and by
813 repeated recovery in separate wells. Selected PCR products were cloned into pVRC8400 to
814 construct heavy chain- and light chain-expressing plasmids for mAb expression and purification.
815

816 mAb and Fab Production

817   mAbs were expressed by cotransfection of Expi293F or Freestyle 293F cells (Thermo
818 Fisher) with heavy chain- and light chain-expressing plasmids according to the cell manufacturer's
819 directions. Following a 6-day incubation, transfection mixtures were pelleted by centrifugation
820 and filtered through 0.22□m Stericup filter units (EMD Millipore). Filtered supernatant was applied
821 to a column containing a 1ml bed of Protein A Sepharose Fast Flow (GE Healthcare, Chicago, IL)
822 equilibrated with Pierce Protein A IgG Binding Buffer (Thermo Fisher). The column was washed
823 with Protein A IgG Binding Buffer, and mAb was eluted with Pierce IgG Elution Buffer (Thermo
824 Scientific) and collected in a 1:10 volume of 1M Tris pH 8 solution. Antibodies were buffer-
825 exchanged in PBS using 10,000 MWCO Amicon Ultra-15 centrifugal filter units (EMD Millipore)
826 over three rounds of spinning.

827   For Fab production, the cleavage site (GLEVLFQGP) for the 3C protease of human
828 rhinovirus (HRV3C) was inserted into the hinge region of the Ab heavy chain expression plasmid.
829 Following expression and purification of mAb, the protein was subjected to HRV3C protease.
830 Products were purified by gel filtration, and Fab fragments were collected for use in assays.
831

832 Biotinylation of mAbs

833   MAbs were biotinylated using EZ-Link Sulfo-NHS-Biotin (Thermo Fisher). Briefly, mAb
834 was concentrated to 2-2.5 mg/ml (200-250□l volume) and mixed with 15□l of 50mg/ml Blue
835 Dextran. MAb:Dextran mixture was applied to a Sephadex G25, NAP-5 column (GE Healthcare)
836 previously washed 5X with 0.1M bicarbonate buffer (100mM $NaHCO_3$, pH 8.4). Antibody was
837 eluted with 0.1M bicarbonate buffer, and the blue fraction containing mAb was collected. EZ Link
838 Sulfo-NHS-Biotin (prepared as a 10mg/ml solution with dimethylsulfoxide) was added at 80□g
839 per 1mg mAb ratio and allowed to mix by rotating for 4 hours at room temperature. Biotinylated
840 mAb was then loaded onto a Sephadex G25, PD-10 desalting column (GE Healthcare) previously
841 washed 5X with a storage buffer containing 10mM Tris, 150mM NaCl, 0.1% NaN_3,pH 8.2. MAb
842 was eluted with storage buffer, and the blue fraction containing biotinylated mAb was collected.
843

844 ELISAs with Recombinant VLPs, gp120 and gp140

845   Briefly, Immulon II plates were coated with either 20x concentrated VLPs (Tong et al.,
846   2012), 5μg/ml recombinant gp120 monomer, or 5μg/ml gp140 trimers overnight at 4°C. Plates
847   were washed with PBS twice and blocked. For VLP ELISA, blocking buffer was 4% BSA in 10%
848   FBS/PBS, and for gp120 or gp140 ELISAs, blocking buffer was 4% milk in 0.05% Tween20/PBS.
849   After 1h of blocking at room temperature and 2 washes with PBS or PBS containing 0.1% Tween
850   20 (PBS-T), serially diluted sera or mAbs were added in the following reaction buffers: 2% BSA
851   in 10%FBS/PBS for VLP ELISA and 2% milk in PBS-T for gp120 or gp140 ELISAs. Plates were
852   incubated for 1 hour at 37°C followed by 2 PBS or PBS-T washes. Species-specific alkaline
853   phosphatase anti-Fc conjugates (Accurate, Westbury, NY) diluted in the corresponding reaction
854   buffers at 1:5,000 were then added and plates were incubated at 37°C for 30 minutes. After a 4X
855   wash with distilled H_2O, 50□l of SigmaFAST p-nitrophenyl phosphate tablets (Sigma, St. Louis,
856   MO) dissolved in distilled H_2O was added and incubated for 1 hour at room temperature in the
857   dark. Reaction was stopped by adding 10□l of 3N NaOH and detected at 405nm.

858   ELISAs using SOSIP gp140 trimers, gp140F, gp120 monomers, and V1V2 scaffolds were
859   performed on Reacti-Bind 96-well polystyrene plates (Pierce). Briefly, plates were coated with
860   2□g/ml of monomer/trimer in PBS overnight at 4°C. Following 6 washes with PBS-T and blocking
861   for 1 hour at 37°C with B3T buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 3.3% FBS, 2%
862   bovine serum albumin, 0.07% Tween 20, and 0.02% thimerosal), 5-fold serially diluted sera or
863   mAbs were added in B3T buffer. After a 1-hour incubation at 37°C and 6 washes with PBS-T,
864   plates were incubated with HRP-conjugated anti-human IgG, Fc□ fragment-specific antibody
865   (Jackson ImmunoResearch, West Grove, PA) diluted 1:10,000 in B3T buffer for 1 hour at 37°C.
866   After 6 washes with PBS-T, SureBlue TMB Substrate (KPL, Gaithersburg, MD) was added,
867   incubated for 10 minutes, and the reaction was stopped with 1N H_2SO_4 before measuring binding
868   at 450nm.

869   Competitive VLP ELISAs was performed similarly to regular VLP ELISAs. Plates were
870   coated, washed and blocked in the same manner. After blocking, plates were washed twice with
871   PBS, and 25□l of competitor antibody was added at a fixed concentration. Following a 10-minute
872   incubation at 37°C, titrated biotinylated or Strep II-tagged mAbs were added and incubated for an
873   additional hour at 37°C. After 2 washes with PBS, conjugates were added as follows: 1:300-
874   diluted streptavidin-alkaline phosphatase (Vector, Burlingame, CA) for biotinylated antibodies or
875   1:500-diluted streptactin-alkaline phosphatase (IBA Life Sciences, Goettingen, Germany) for
876   Strep II-tagged antibodies. Plates were washed with distilled H_2O 4 times, 50□l of SigmaFAST
877   p-nitrophenyl phosphate tablets (Sigma) dissolved in distilled H_2O water was added, and plates

878 were incubated for 1 hour at room temperature in the dark. Reaction was stopped by adding 10□l

879 of 3N NaOH before measuring binding at 405nm.

880

Measuring Probe Binding to mAb-Coated Beads

882 CompBead Anti-Mouse Ig, □□□□□□□□□□□□□□□□□□□□□□□□□□ (BD Biosciences)

883 were coated with mAbs for antigenic analysis of HIV-1 Env proteins and trimers. Briefly, 1ml of

884 particles (beads) was pelleted at 1000Xg for 5 minutes, and supernatant was aspirated. Beads

885 were resuspended in 1ml 0.5% bovine serum albumin (BSA) in PBS. 1□g of Purified Mouse Anti-

886 Human Ig □ Light Chain antibody (BD Biosciences) was added and allowed to incubate for 30

887 minutes at room temperature. 2ml of 0.5% BSA/PBS was added, beads were pelleted at 1000Xg

888 for 5 minutes, and supernatant was aspirated. Beads were resuspended in 1ml of 0.5%

889 BSA/PBS, and 1□g of mAb was added, mixed, and incubated for 30 minutes at room temperature.

890 2ml of 0.5% BSA/PBS was added, beads were pelleted at 1000Xg for 5 minutes, and

891 supernatants were aspirated. Beads were resuspended in 1ml 0.5% BSA/PBS for short-term

892 storage of up to 1 week at 4°C. For staining, 50□l of antibody-coated beads were mixed with

893 different amounts of streptavidin-conjugated HIV-1 Env protein or trimer, incubated with 30

894 minutes at 4°C, and washed with 3ml of PBS. Beads were pelleted at 1000Xg for 5 minutes,

895 supernatants were aspirated, and beads were resuspended in 300-500□l PBS for flow cytometric

896 analysis.

897

Neutralization Assays Using TZM-bl Cells

899 10□l of five-fold serially diluted mAbs or patient serum in cDMEM was incubated with 40ul

900 of diluted HIV-1 Env-pseudotyped virus and incubated for 30 minutes at 37°C in a 96-well tissue

901 culture plate. 20 □l of TZM-bl cells (10,000 cells/well) with or without 70□g/ml DEAE-Dextran

902 was then added and incubated overnight at 37°C. Each experiment plate also had a column of

903 cells only (no Ab or virus) and a column of virus only (no Ab) as controls for background TZM-bl

904 luciferase activity and maximal viral entry, respectively. The following day, all wells received

905 130□l of fresh cDMEM and were incubated overnight at 37°C. The following day, media was

906 aspirated from wells, and 50ul of 1X Cell Culture Lysis Reagent (Promega) in distilled H_2O was

907 added to cells. Lysates were shaken at 600RPM for 15 minutes, and 30□l of lysate was

908 transferred to OptiPlate black plates (Perkin Elmer). Luminometry was performed on a Perkin

909 Elmer Model #1420-061 luminometer using the Promega Luciferase Assay System reagent

910 (reconstituted in buffer provided with the kit, equilibrated to room temperature). Percent

911 neutralization is determined by calculating the difference in average RLU between virus only wells

912 (cells + virus column) and test wells (cells + serum/Ab sample + virus), dividing this result by the
913 average RLU of virus only wells (cell + virus column) and multiplying by 100. Background is
914 subtracted from all test wells using the average RLU from the uninfected control wells (cells only
915 column) before calculating the percent neutralization. Neutralizing serum antibody titers are
916 expressed as the serum dilution/antibody concentration required to achieve 50% neutralization
917 and calculated using a dose-response curve fit with a 5-parameter nonlinear function.
918
919 Neutralization Assays Using CF2 Cells
920   Neutralization assays using CF2 cells were performed as described previously **(Crooks
921 et al., 2015)**. On the day prior to the assay, 96 well plates were seeded with 200￼l of
922 CF2Th.CD4.CCR5 cells at $1x10^5$/ml per well. On the day of the assay, virus was incubated with
923 serially diluted mAbs or serum for 1 hour at 37 °C. The virus:antibody/serum mixture was then
924 added to plates seeded with CF2 cells, spinoculated at 300Xg for 15 minutes, and incubated for
925 3 days at 37 °C. Luciferase activity was measured using the Promega Luciferase Assay System
926 reagent.
927
928 HEp-2 Cell Staining
929   Autoreactivity staining assays were performed on HEp-2 cells per the manufacturer
930 recommendations (Zeus Scientific, Branchburg, NJ). MAbs were diluted to 50 and 25 ￼g/ml using
931 SAVe Diluent. 20￼l of the appropriate dilution was coated onto cells fixed on the slide and
932 incubated for 30 minutes at room temperature in a humidified chamber. Slides were rinsed in 1X
933 PBS, washed twice in 1X PBS in Coplin jars for 3-5 minutes and then stained with 20￼l of FITC-
934 conjugated secondary antibody for 30 minutes in a humidified chamber. Slides were rinsed in 1X
935 PBS, washed twice in 1X PBS in Coplin jars for 3-5 minutes and mounted with 15￼l of mounting
936 media per well and a cover glass (Thermo Scientific). Slides were imaged on a Nikon Eclipse
937 E800 microscope at 20X in the RGB mode for 2 seconds using SPOT 5.0 software (SPOT
938 Imaging, Sterling Heights, MI). VRC01, 4E10, VRC07-523 and VRC07-G54W were used as
939 control mAbs for staining and given a score of 0, 1, 2 and 3 respectively based on their staining
940 intensity. Test antibodies were assigned scores based on visual comparisons of staining intensity
941 to the control antibodies.
942
943 Cardiolipin ELISA
944   MAb binding to cardiolipin was tested by ELISA per the manufacturer's protocol (Inova
945 Diagnostics, San Diego, CA). Starting at 100￼g/ml, mAbs were tested in a 3-fold series. Assays

946   were validated using positive and negative controls and standards provided in the kit. OD values
947   were converted to IgG anti-phospholipid (GPL) units by linear regression. 4E10 and VRC01 were
948   used as additional positive and negative controls. Cardiolipin binding was scored as follows: no
949   binding for <15 GPL, indeterminate for 15-20 GPL, low positive for 20-80 GPL and high positive
950   for >80 GPL.
951
952   Genetic Clustering and Phylogenetic Analysis
953   MAb sequences were aligned by ClustalOmega, followed by ClustalW-Phylogeny and
954   Dendroscope **(Huson et al., 2007)** to generate and display the phylogenetic tree. MAb lineages
955   were inferred using *Cloanalyst* (http://www.bu.edu/computationalimmunology). Briefly, DNA
956   Maximum Likelihood (DNAML) from the Phylip molecular evolution suite was used to find the
957   maximum likelihood tree using the γ and κ chains together, with mutation rates differing by chain
958   and varying by site. The inferred ancestor (IA) and intermediates were determined by computing
959   the Bayesian posterior probability over all possible sequences representing unmutated
960   immunoglobulin variable-region gene rearrangements.
961
962   Blue Native PAGE-Western Blot
963   VLPs were solubilized in 0.12% Triton X-100 in 1 mM EDTA/1.5 M aminocaproic acid with
964   a protease inhibitor cocktail (P-2714; Sigma). An equal volume of 2X sample buffer (100 mM
965   morpholinepropanesulfonic acid, 100 mM Tris-HCl, pH 7.7, 40% glycerol, and 0.1% Coomassie
966   blue) was added. Samples were then loaded onto a 4-12% Bis-Tris NuPAGE gel (Thermo Fisher)
967   and separated at 4°C for 3 hours at 100V. The gel was then blotted onto polyvinylidene difluoride
968   and destained with a solution containing 35% methanol and 10% acetic acid followed by 100%
969   methanol until blue stain was removed. The blot was blocked using 4% nonfat milk in PBS for 1
970   hour at room temperature and probed with mAbs 2G12, b12, 39F, 2F5 and 4E10 at 1µg/ml each.
971   The blot was developed with anti-human Fc alkaline phosphatase conjugate (Jackson
972   ImmunoResearch, West Grove, PA) diluted 1:5,000 in 2% milk in TBS-T followed by SigmaFast
973   BCIP/NBT substrate (Sigma).
974   In BN-PAGE "shift" assays **(Moore et al., 2006; Tong et al., 2012)** mAbs were incubated
975   at 37°C for 1 hour with 12.5□g gp120 equivalents of Env that was liberated from VLPs by adding
976   1% Triton X-100. Complexes were then resolved by BN-PAGE-Western blot as above and probed
977   with a cocktail of biotinylated mAbs (b12, 2G12 and 4E10) followed by a streptavidin-alkaline
978   phosphatase conjugate and BCIP/NBT.
979

<u>Negative-stain EM</u>

980
981 VRC38 IgG or monovalent Fab was incubated overnight in Tris-buffered saline (TBS)
982 containing 50mM Tris, 150mM NaCl, pH 7.4) at room temperature with BG505 or B41 SOSIP.664
983 gp140 trimers **(Pugach et al., 2015)** at a 10X molar excess of mAb. Samples were then diluted
984 to ~0.01mg/ml in TBS and applied to a plasma-cleaned carbon-coated Cu400 mesh grid (Electron
985 Microscopy Sciences, Hatfield, PA) for about 10 seconds. Nano-W stain (NanoProbes, Inc.,
986 Yaphank, NY) was applied for 7 seconds, blotted with filter paper, and a fresh drop applied for an
987 additional 15 seconds before blotting. Image collection and data processing was performed as
988 described elsewhere **(de Taeye et al., 2015)** on either an FEI Tecnai T12 microscope (2.05
989 Å/pixel; 52,000X magnification) or FEI Talos microscope (1.57Å/pixel; 92,000X magnification). 2D
990 class averages for these datasets and other data **(Doria-Rose et al., 2014; Julien et al., 2013;**
991 **Sok et al., 2014)** were generated by Iterative MSA/MRA **(Ogura et al., 2003)** or SPARX ISAC
992 methods **(Yang et al., 2012).** Figures were created using UCSF Chimera **(Petterson et al., 2004)**.

993

<u>Structural Analysis</u>

994
995 To form VRC38-1FD6-WITO-V1V2 complexes, 3mg of purified VRC38.01 HRV3C mutant
996 IgG was bound to 750 ml Protein A Plus Agarose (Pierce) in a disposable 10ml column. 10mg of
997 V1V2 scaffold, produced in GnTI- cells, was then added. Misfolded or improperly glycosylated
998 scaffold remained unbound to Ab and was flushed with 5 column volumes of PBS. The column
999 was capped and 20□l of HRV3C protease at 2U/□l was added in 1ml of PBS. After 2 hours at
1000 room temperature, the resin was drained, the eluate collected and passed over a 16/60 S200
1001 column in buffer containing 5mM HEPES 7.5, 50mM NaCl, and 0.02% NaN$_3$. VRC38.01–1FD6-
1002 WITO-V1V2 complex fractions were pooled and concentrated to 10mg/ml.
1003 Samples of monovalent VRC38.01 Fab, both unliganded and in complex with 1FD6-
1004 WITO-V1V2, were screened for crystallization using 572 conditions from Hampton, Wizard and
1005 Precipitant Synergy screens using a Cartesian Honeybee and a mosquito crystallization robot
1006 with 0.1□l of reservoir solution and 0.1□l of protein solution per condition. Crystals for VRC38.01
1007 Fab obtained in 14% PEG400, 13% PEG8000, 0.1M Tris pH 8.5, 0.1M MgCl$_2$ were flash-frozen
1008 in liquid nitrogen with no cryoprotectant. Crystals of VRC38.01–1FD6-WITO-V1V2 obtained in
1009 27% isopropanol, 0.1M imidazole pH 6.5 and 10% PEG8000 were flash-frozen in liquid nitrogen
1010 in mother liquor supplemented with 20% 2-methyl-2,4-pentanedial (MPD). Data were collected at
1011 1.00Å using the SER-CAT beamline ID-22 of the Advanced Photon Source, Argonne National
1012 Laboratory (Lemont, IL).

1013 Diffraction data were processed with the HKL2000 suite 6 (HKL Research, Inc.,
1014 Charlottesville, VA). A molecular replacement solution for unliganded VRC38.01 obtained with
1015 Phenix (www.phenix-online.org) contained one Fab molecule per asymmetric unit in space group
1016 $P2_122_1$. The structure of the complex contained one Fab bound to a 1FD6–WITO-V1V2 monomer
1017 in space group $P3_221$. Model building was carried out using COOT software (https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/)
1018 lmb.cam.ac.uk/personal/pemsley/coot/), and was refined with Phenix. Final data collection and
1019 refinement statistics are shown in supplemental Table 1. The Ramachandran plot determined by
1020 Molprobity (http://molprobity.biochem.duke.edu) shows 98.4% of all residues in favored regions
1021 and 100% of all residues in allowed regions for the unliganded Fab structure and 92.8% of all
1022 residues in favored regions and 99.2% of all residues in allowed regions for the complex structure.
1023
1024

1025 Biolayer Interferometry
1026 BG505.T332N.His8x SOSIP.664 was coated onto a His1K Octet biosensor surface
1027 (ForteBio, Menlo Park, CA) at 10□g/ml for 60 seconds, followed by a baseline for 60 seconds and
1028 an association step with saturating concentrations of various mAbs (50□g/ml of IgG for 30 minutes
1029 or 50□g/ml Fab fragments for 45 minutes). Response values from the last 30 seconds of the
1030 association step were averaged to obtain the saturation binding. Mean binding response and
1031 standard deviations from 3 independent experiments were plotted against the known number of
1032 binding sites occupied by each mAb or mAb cocktail by regression analysis to generate a standard
1033 curve for trimer occupancy.
1034

1035 Surface Plasmon Resonance Analysis
1036 Affinities and kinetics of binding of VRC38.01 Fab and IgG to BG505 DS-SOSIP.664
1037 soluble trimer were assessed by surface plasmon resonance on a Biacore S-200 (GE Healthcare)
1038 at 25°C with buffer HBS-EP+ (10mM HEPES, pH 7.4, 150mM NaCl, 3mM EDTA, and 0.05%
1039 surfactant P-20). Ab was first immobilized onto two flow cells on a CM5 chip at ~800 response
1040 units (RU) with standard amine coupling protocol (GE Healthcare). PGT145 IgG was immobilized
1041 as a control to ensure trimer fidelity with BSA immobilized in the reference channel. Trimer, at
1042 two-fold dilutions starting from 40nM were injected at a flow rate of 50□□□□□ for 4 minutes
1043 and allowed to dissociate for 6 minutes. The cells were regenerated with 100□l injections of 3.0M
1044 $MgCl_2$ at a flow rate of 50□l/min. Sensorgrams of the concentration series were corrected with
1045 corresponding blank curves and fitted globally with Biacore S200 evaluation software (GE

1046   Healthcare) using a 1:1 Langmuir model of binding to highlight the apparent change in affinity

1047   from Fab to IgG.

1048

1049   <u>Isothermal Titration Calorimetry</u>

1050   Isothermal titration calorimetry (ITC) was performed using the ITC200 microcalorimeter

1051   system (MicroCal Inc., Malvern, Worcestershire, United Kingdom). Proteins were dialyzed against

1052   filtered PBS before use. The concentration of BG505 native, flexibly linked (NFL) trimer **(Sharma**

1053   **et al., 2015)** in the sample cell was approximately 25□M, and that of VRC38.01 Fab in the syringe

1054   was approximately 5□M. Reactions were carried out at 37°C, as previously described **(Wu et al.,**

1055   **2010)**. Briefly, BG505 NFL trimer was titrated to saturation by stepwise addition of 2□l ligand in

1056   the syringe at 120-second intervals at 37°C. The heat evolved upon each injection was obtained

1057   from the integral of the calorimetric signal. The values of enthalpy (□H) and entropy (□S) were

1058   obtained by fitting the data to a nonlinear least-squares analysis with Microcal ORIGIN software

1059   using a single-site binding model.

1060

1061   <u>Neutralization Fingerprinting Analysis</u>

1062   Published neutralization data for a set of ~200 donor plasma samples **(Hraber et al., 2014)**

1063   was analyzed by using a next-generation neutralization fingerprinting algorithm **(Doria-Rose et**

1064   **al., 2017)**. For a given sample, the approach compares a polyclonal neutralization pattern of a set

1065   of diverse viral strains to the neutralization patterns (or fingerprints) of a reference set of broadly

1066   neutralizing mAb specificities, to obtain an estimate of the contribution of each of the reference

1067   specificities to polyclonal neutralization **(Doria-Rose et al., 2017; Georgiev et al., 2013)**.

1068   Reference broadly neutralizing antibodies were used as previously described **(Pancera et al.,**

1069   **2014)**, with the VRC38 fingerprint as a separate category. We applied computational quality

1070   control metrics for filtering out plasma samples for which the predictions were deemed unlikely to

1071   be accurate. Specifically, these included metrics for: (i) predicting the presence of dominant novel

1072   specificities and (ii) computing a confidence score associated with the computational predictions

1073   for each given sample **(Doria-Rose et al., 2017)**. Through this process, the initial set of samples

1074   was reduced to 80 samples, which were used for the analysis of Ab specificity frequency. Samples

1075   were predicted to have between 1 and 3 specificities from the reference set, and the overall

1076   frequency of observing the different reference specificities or pairs of reference specificities were

1077   analyzed.

1078

1079

1080 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1081   For all mAb/serum pseudovirus neutralization assays (Figures 1E, 6D, and 6E;
1082 Supplemental Figures S2A, S3B, S4A, S5B, S5F, S6A, S7E, and S7F; and Supplemental Tables
1083 1, 3, and 5), data were fitted to a 5-parameter asymmetric nonlinear regression model to obtain
1084 the IC50, or concentration of mAb/dilution of serum needed to obtain 50% neutralization against
1085 a given pseudovirus. For neutralization assays in which a fold-change in IC50 imparted by a
1086 particular virus mutant, virus treatment, or binding stoichiometry (e.g., IgG vs. Fab) was reported
1087 (Figures 6D and 6E; Supplemental Figures S4A, S5B, S5F, and S7E; and Supplemental Tables
1088 3 and 5), the IC50 obtained for one virus/assay condition was divided by the IC50 obtained for
1089 the other virus/assay condition, as indicated in the figure legends and *y*-axes of data graphs. All
1090 neutralization assays were repeated at least 2 times, and data shown are from representative
1091 experiments.

1092   Multiple binding assays were performed to investigate VRC38.01 binding to HIV-1 Env
1093 trimers and monomers. All ELISAs (Figures 2A, 2B, and 2C; and Supplemental Figures S2C,
1094 S2D, S2F, and S3A) were repeated at least two times, and curves shown are from representative
1095 experiments. BLI measurements (Supplemental Figure S4D) were taken over 3 independent
1096 experiments, and the means and standard deviations of binding values were plotted. Binding
1097 stoichiometry of VRC38.01 IgG and Fab were interpolated from a standard linear regression
1098 equation (indicated in the plots) derived from the BLI response data (nm) plotted against number
1099 of known IgG/Fab binding sites per trimer. Association and dissociation kinetics derived from
1100 SPR data (Supplemental Figure S4B) were fitted to a 1:1 Langmuir model using Biacore S200
1101 software, and data from a representative of 3 independent experiments is shown. For ITC,
1102 enthalpy and entropy values were interpolated from a nonlinear least-squares analysis using
1103 Microcal ORIGIN software using a single-site binding model, and results shown are a
1104 representative of 3 independent experiments.

1105

1106 **DATA AND SOFTWARE AVAILABILITY**

1107   The sequences for VRC38.01-VRC38.14 heavy chains and VRC38.01-VRC38.11 light
1108 chain have been deposited in GenBank under ID codes KY905214-KY905227 and KY905228-
1109 KY905238, respectively. The crystal structures for unliganded N90-VRC38.01 Fab and V1V2-
1110 scaffold complex have been deposited in PDB under ID codes 5EWI and DDD, respectively.

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