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

Most HIV-1-specific neutralizing antibodies isolated to date exhibit unusual characteristics that complicate their elicitation. Neutralizing antibodies that target the V1V2 apex of the HIV-1 envelope (Env) trimer feature unusually long protruding loops, enabl them to penetrate the HIV-1 glycan shield. As antibodies with loops of requisite length are created through uncommon recombination events, an alternative mode of apex binding has been sought. Here, we isolated a lineage of Env apex-directed neutralizing antibodies, N90-VRC38.01-11, using virus-like particles and conformationally stabilized Env trimers as B cell probes. A crystal structure of N90-VRC38.01 with a scaffolded V1V2 revealed a binding mode involving side-chain to side-chain interactions that reduced the distance the antibody loop must traverse the glycan shield, facilitating V1V2 binding via a non-protruding loop. The N90-VRC38 lineage identifies a solution for V1V2apex binding that provides a more conventional B cell pathway for vaccine design.

KEY WORDS

antibody, B cell ontogeny, bnAb, glycan shield, HIV, neutralization, trimer, vaccine, VLP

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 1,135$Å^2$ 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

290 even less on PGT145, which neutralized a N130 glycan-bearing DU422.01 mutant more
291 effectively (Figure 5D). These findings are consistent with the latter bnAbs targeting epitopes
292 closer to the trimer vertex. Reciprocal removal of the N130 glycan increased VRC38.01 sensitivity
293 of BaL.26 and 25711-2.4 by >40-fold and CAAN.A2 by >7-fold (Figure 5E). VRC38.01 achieved
294 47.5% neutralization of strain DU172.17 at 50 μg/ml, but did not neutralize the parent virus (Figure
295 5E). Again, the effect of removing glycan was less pronounced PG9 and PGT145, and in fact
296 reduced PG9 sensitivity of the 25711-2.4 strain (Figure 5E). Autologous N90 donor viral
297 sequences (Wu et al., 2012) exhibited key contacts for VRC38 but had a sequon at N130 (3) and
298 were therefore VRC38.01-resistant. Upon N130D mutation, VRC38.01 reached 50%
299 neutralization for 3 of viruses (Figure S7E). For the other 5 viruses tested, we detected increased
300 VRC38.01 sensitivity that did not reach 50% at 100 μg/ml (Figure S).

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

311

312 **VRC38.01 olerates arge annose ranches**

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

321

322 **Ontogen VRC38 eutraliz**

323 A phylogenetic tree of all 11 lineage members was generated, rooted by a common
324 inferred ancestor (IA, Figure 6A). A variant with germline V-gene and J-gene reverted heavy (gH)
325 and light (gL) chains, but maintaining the mature CDRH3, was unable to neutralize (Table S1).
326 However, hybrids consisting of mixed mature and reverted heavy and light chain partners, gHL
327 and HgL, neutralized 19 and 18 strains of a 208-member panel, respectively (). Fourteen strains
328 were sensitive to both hybrids, while others were neutralized by only one hybrid, indicating that
329 both chains contribute to neutralization. When both the gHL and HgL Abs were effective, HgL
330 potency was generally stronger, suggesting that the mature heavy chain plays a dominant role ().
331 Overall, this suggests that VRC38 can neutralize ~9% of heterologous strains with no maturation
332 of the V-gene of one chain.
333 VRC38 heavy and light chain maturation was modeled in Figure 6 and Table S6,
334 respectively. S31D mutation likely occurred early in the VRC38 heavy chain (Figure 6A and boxed
335 starting at sequence I1 in Figure 6B) and forms a salt bridge with a 97% conserved basic residue
336 at position 168 (Figure 6C). However, data above (Figure 1E) suggests that, unlike other V1V2
337 NAbs, this contact is not essential (Figure SB). Indeed, a D31S mutant modestly reduced
338 neutralization of 11 of the 14 strains tested (Figure 6D). Two exceptions were BG1168.01 was
339 more sensitive to the D31S mutant and ZA012.29 was insensitive to either the parent mAb o
340 D31S mutant.
341 VRC38.01 share a branch with broad lineage members VRC38.07 and .06, to a S97E
342 mutation acquired at intermediate I3 (Figure 6A-C), that forms a salt bridge with K171 of strand C
343 (Figure 6C). E97S reversion reduced VRC38.01 neutralization to a larger degree than the D31S
344 mutant. The two reversions together led to loss in neutralization, suggesting that both contacts
345 contribute to VRC38.01 neutralizing activity (Figure 6D). VRC38.09, which is almost as broad as
346 the three members of the VRC38.01 branch but almost 3-fold lower in potency, lacks the S97E
347 mutation. However, a S97E mutant increased its potency agains 12 of the 14 strains (Figure 6E).
348 Exception were uniquely VRC38.09-sensitive strain X1632.S2.B10 (Figure 1E) and the
349 BG1168.01 strain. The behavior of the latter mirrored the effect of the reciprocal mutation on
350 VRC38.01 (Figure 6D and E). Overall, VRC38.09's breadth developed independently from other
351 clones and the sensitivity of some strains to this variant depend on different somatic mutations.
352

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

354 To analyze VRC38-like bnAb prevalence, we used neutralization fingerprinting (Doria-
355 Rose et al., 2017; Georgiev et al., 2013) to delineate bnAb specificities in a cohort of HIV-1
356 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.

362 **DISCUSSION**

363

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

373 Our fingerprint analysis suggested that VRC38-like Abs may be as prevalent as PGT128-
374 like or PG9-like Abs. We are now mapping donor sera with VRC38-like signals and probing their
375 B cells. Given its common features, VRC38-like NAbs might have been expected to be even more
376 . However, N130 glycan is found in >60% of circulating virusesthe single largest regulator of
377 VRC38's breadth prevent VRC38-like bnAb development or facilitate escape. Accommodating
378 glycan might therefore be a route to improved VRC38 breadth. We are now assessing additional
379 donors and modifying B cell probe strategy. For example, in a variation of the strategy in Figure
380 1C, B cells could be gated for positive binding to bearing a N130 glycan knock in mutation. It may
381 also be useful to interrogate the N90 donor for VRC38 signatures by NGS and to probe samples
382 from other donors that exhibit VRC38 fingerprint signatures. In a vaccine setting, N130 clash could
383 be addressed using priming immunogens bearing a N130 "glycan-hole" (Crooks et al., 2015) to
384 promote VRC38-like NAb development and boosting with N130 glycan-containing immunogens
385 to select for clones that can accommodate the glycan and thereby acquire greater breadth.
386 Feasibility is supported by the observations that CD4bs-specific bnAb N6 evolved greater breadth
387 by accommodating V5 loop glycans (Huang et al., 2016) and that the PGT121 family gained
388 breadth by accommodating the N137 glycan (Garces et al., 2015).

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

395 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.

456 **AUTHOR CONTRIBUTIONS**

457

458 Conceptualization, EMC, JG, NAR, PDK, JRM, and JMB; Methodology, EMC, JG, ETC, TT, NDR,
459 TBK, ABW, ISG, JMB, JRM, and JMB; Software, TBK and ISG; Validation, RTB and MKL;
460 Investigation, EMC, JG, NAR, ETC, KO, TT, JL, RN, GO, DRA, MA, AKB, YF, SO, CO, and TBK;
461 Resources, ETC, TT, XC, AD, MGJ, MP, MC, TJH, and RTW; Writing – Original Draft, EMC, JG,
462 and JMB; Writing – Review & Editing, EMC, JG, PDK, JRM, and JMB; Visualization, EMC, JG,
463 GO, ISG, and JMB; Supervision, ABW, ISG, PDK, JRM, and JMB; Project Administration, EMC,
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465

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467

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476 **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 Human Subjects
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 Cell Lines

Human embryonic kidney (HEK)-derived 293T, HEK 293S N-acetylglucosaminyltransferase I-negative (GnTI-), and HeLa-derived TZM-bl cells were maintained in complete Dulbecco's Modified Eagle Medium (herein referred to as cDMEM) containing high-glucose Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher, Waltham, MA), 1X Penicillin-Streptomycin (Pen Strep, Thermo Fisher) and 10% fetal bovine serum (FBS, Gemini Bio Products, West Sacramento, CA) at 37ºC/5% CO_2. Canine thymus-derived CF2Th.CD4.CCR5 cells were maintained in DMEM containing 1X Pen Strep, L-glutamine, 500 □g/ml G418, 150 □g/ml Hygromycin, and 10% FBS at 37ºC/5% CO_2. FreeStyle 293F and Expi293F cells (both Thermo Fisher) were maintained in Freestyle 293 Expression Medium and Expi293 Expression Medium, respectively, at 37ºC/10% CO_2 with shaking at 120 RPM. Human Burkitt's B cell lymphoma-derived Ramos cells were maintained in either DMEM containing 15% FBS and 1X Pen Strep or Roswell Park Memorial Institute 1640 medium (RPMI 1640, Thermo-Fisher) containing 10% FBS and 1X Pen Strep at 37ºC/5% CO_2. In some cases, Ramos cells were engineered to stably express mature B cell receptors (BCR) IgM versions of VRC01, PGT145, and PGT128.

METHOD DETAILS

Anti-HIV-1 Env Monoclonal Abs

MAbs were obtained from the NIH AIDS Reagent Repository or from their originators. Further information can be found in the HIV Molecular Immunology Database (http://www.hiv.lanl.gov/) and in ref **(Burton and Hangartner, 2016)**. MAbs included the following (originators given in parentheses): 2G12 (H. Katinger), directed to a glycan cluster on gp120 ; 39F and CO11 (J. Robinson), directed to the gp120 V3 loop; b12 (D. Burton), VRC01, VRC07-G54W, VRC13 (J. Mascola) and HJ16 (A. Lanzavecchia) directed to epitopes that overlap the CD4bs; PGT121, PGT125 and PGT128 (D. Burton) directed to epitopes involving the base of the V3 loop of gp120 and the N332 glycan; PG9, PG16, PGT145, PGDM1400 (D. Burton), CH01 and CH03 (B. Haynes) and CAP256.01-33 (J. Mascola), directed to quaternary, glycan-dependent V1V2 loop-directed epitopes; PGT151 (D. Burton), 35O22 (M. Connors), and 8ANC195 (M. Nussenzweig), directed to the gp120/gp41 interface; 10E8 (M. Connors), 2F5 and 4E10 (H. Katinger), directed to the gp41 membrane-proximal ectodomain region (MPER).

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 Ramos-IgM Cell Line Production

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

Biosciences, San Jose, CA) and anti-human-IgM (Biolegend, San Diego, CA) by flow cytometry and sorted using a FACS Aria (BD Biosciences). Cells were amplified, assessed for BCR expression as before, and resorted for highest-staining cells if needed.

<u>VLP Production</u>

VLPs were produced by co-transfecting 293T or Freestyle 293F cells with plasmids expressing Env, Gag, and Rev **(Crooks et al., 2015)**. Fluorescent VLPs were produced by also cotransfecting plasmids pGag-GFP or pGag-RFP. Two days later, supernatants were collected, filtered, precleared by centrifugation at 450Xg for 5 minutes, and concentrated either by tangential flow filtration or by centrifugation at 50,000Xg in a Sorvall SS34 rotor. "Trimer-VLPs" were made by digesting with 1□l each of 1mg/ml proteinase K, subtilisin, and trypsin, and 1□l of 2mg/ml chymotrypsin **(Crooks et al., 2011; Tong et al., 2012).** pNL4-3.Luc.R-E- based fluorescent VLPs were inactivated using 1mM aldrithiol (AT-2) before use as baits **(Crooks et al., 2015)**. RFP-labeled “Bald-VLPs” were produced by co-expressing pMV-2024 (expressing SIV Gag) and pGag-RFP plasmids.

<u>Soluble Env Protein Production</u>

Trimeric Env constructs were expressed in various types of 293 cells and then purified by previously published methods **(Bhiman et al., 2015; Doria-Rose et al., 2015; Stewart-Jones et al., 2016)**. For BG505.T332N.SOSIP proteins used for flow cytometric cell sorting, Avi-tagged proteins were biotinylated using the BirA Biotin-Protein Ligase Bulk Reaction Kit (Avidity, LLC, Aurora, CO) under the following conditions and reagents from the kit: 25□l of 10X Biomix A, 25□l of 10X Biomix B, 20□g of BirA protein, and 180□l of Avi-tagged SOSIP protein, incubated for 5 hours at 30ºC with constant agitation. The mixture was buffer exchanged into SOSIP buffer containing 5mM HEPES, 150mM NaCl, and 0.02% NaN_3 in 30,000 MWCO Amicon Ultra-15 centrifugal filter units (EMD Millipore) over 5 rounds of spinning at 3000Xg, 4ºC. Biotinylated SOSIP was then conjugated with streptavidin, R-phycoerythrin (SA-PE, Thermo Fisher) at a 1.17 SA-PE:SOSIP weight ratio (determined empirically). Briefly, biotinylated SOSIP was mixed with 1/5 the final volume of SA-PE and incubated for 20 minutes at 4ºC with gentle rotation. Subsequent 1/5 increments of SA-PE were then added with 20-minute incubations in between each addition. APC-conjugated YU2 gp140 foldon (gp140F) protein was biotinylated and conjugated to SA-allophycocyanin (SA-APC, Thermo Fisher) in the same manner at a 1.8 gp140F:SA-APC weight ratio (determined empirically)

V1V2 regions of various strains (residues 126-196, HXB2 numbering) were scaffolded onto a core variant of protein G (PDB 1FD6) and were produced in HEK 293S GnTI- cells and purified over Ni-NTA superflow resin (Qiagen, Hilden, Germany), then digested with HRV3C (Novagen, Darmstadt, Germany) and purified over a 16/60 S200 size exclusion column **(McLellan et al., 2011; Ross et al., 2001)**.

HIV-1 Env-pseudotyped Virus Production

Pseudoviruses for use in TZM-bl neutralization assays were produced in 293T cells by cotransfection of a pSG3ΔEnv backbone plasmid and a full HIV-1 Env gp160-encoding plasmid (Li et al., 2005). Briefly, $2X10^6$ cells in 20ml cDMEM were seeded in T75 flasks the day prior to cotransfection. For transfection, 40μl of FuGene 6 reagent (Promega, Fitchburg, WI) was diluted into 800μl of room-temperature Opti-MEM I reduced serum medium (Thermo Fisher), followed by addition of 10μg of pSG3ΔEnv backbone plasmid. 3.3μg of HIV Env plasmid was then added to the mixture, mixed, and incubated for 30 minutes at room temperature. Transfection mixture was then added to media of previously seeded 293T cells in the T75 flask and then distributed evenly on cells. The following day, media was replaced with 20ml fresh cDMEM. Virus was harvested the following day by filtering cell supernatants with 0.45μm Steriflip units (EMD Millipore, Darmstadt, Germany) and aliquotted.

Pseudoviruses for use in CF2 cell assays were produced in 293T cells by cotransfecting pNL4.3.Luc.R-E backbone plasmid and Env-expressing pCAGGS plasmid using polyethylenimine (PEI) Max (Polysciences, Warrington, PA). Briefly, $5X10^6$ cells were seeded on a 15cm tissue culture dish one day prior to transfection. On the day of transfection, 10μg of pNL4.3.Luc.R-E and 10μg of Env plasmid were mixed in 2ml of serum-free DMEM. 40μl of 1mg/ml PEI Max (DNA:PEI ratio 1:2) was added and incubated at room temperature for 30 minutes. 11ml of cDMEM was then added to the transfection mixture. 293T cells were washed with PBS, and transfection mixture was added to the cells and distributed evenly. Following a 2-hour incubation at 37°C, culture dishes were washed with PBS, and 25ml of cDMEM was added to the cells for a 48-hour incubation at 37°C. Following incubation, virus was harvested by pelleting cell culture supernatant at 450Xg and filtering supernatant through a 0.8μm filter.

For pseudoviruses containing point mutations, mutations were introduced into full HIV-1 Env-encoding plasmids using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's directions. For pseudoviruses produced under conditions containing kifunensine, 25μM kifunensine was added at the time of plasmid cotransfection and replenished during the media change.

744

745 B Cell Sorting of Env Trimer-specific Clones

746 Frozen PBMCs (~$5X10^7$ cells) were thawed in cDMEM containing 50 U/ml of benzonase
747 (Novagen). For flow cytometric sorting, B cells were first enriched by negative magnetic bead
748 selection using the Human B Cell Isolation Kit II (Miltenyi Biotec, San Diego, CA), then washed in
749 PBS, resuspended in LIVE/DEAD® Fixable Violet Dead Cell Stain (Thermo Fisher) and incubated
750 for 30 minutes at 4°C. Cells were washed once in PBS and multicolor staining was performed
751 using a panel of fluorophore-labeled mAbs directed to CD3 (APC-Cy7, clone SK7), CD8 (Brilliant
752 Violet 711, clone RPA-T8), CD14 (Brilliant Violet 605, clone M5E2), CD19 (PE-Cy7, clone HIB19)
753 and human IgG (fluorescein isothiocyanate [FITC] or Alexa Fluor 680, both clone G18-145). All
754 mAbs were obtained from BD Biosciences except for those directed to CD8 and CD14 (Biolegend)
755 and the anti-IgG-Alexa 680 Ab (custom-conjugated at the Vaccine Research Center, National
756 Institutes of Health, Bethesda, MD). Cells were also stained with fluorophore-tagged VLPs,
757 BG505 SOSIP trimers or YU2 gp140F. All staining was performed at 4°C for 30 minutes, followed
758 by two PBS/10% FBS washes and resuspension in PBS. Cells labeled as $CD3^-/CD8^-/CD14^-$
759 $/CD19^+/IgG^+/GFP^+/RFP^-$ memory B cells were gated for VLP stains, or as $CD3^-/CD8^-/CD14^-$
760 $/CD19^+/IgG^+$/BG505 SOSIP trimer$^+$/YU2 gp140F$^-$ for BG505 SOSIP trimer stains. Desirable cells
761 were singly index-sorted on a FACS Aria sorter (BD Biosciences) into 96-well PCR plates
762 (Denville Scientific, Holliston, MA) containing 20□l/well lysis buffer consisting of 1U/□l RNAse
763 OUT (Thermo Fisher), 0.3125% Igepal CA-630, 1X SuperScript III First-Strand Buffer and 6.25
764 mM dithiothreitol (DTT) provided with the Superscript III Reverse Transcriptase kit (Thermo
765 Fisher). Data was collected using FACSDiva software (BD Biosciences). Indexed sort data was
766 analyzed using FlowJo software (FlowJo, LLC, Ashland, OR).

767

768 Single Cell RT-PCR of Ig Genes

769 RT-PCR amplification of IgG heavy and light chain genes was performed as described
770 previously **(Doria-Rose et al., 2015)**. 96-well plates containing single sorted cells were initially
771 frozen at -80°C and thawed to maximize liberation of cellular mRNA. Total mRNA in each well of
772 the 96-well plates was reverse transcribed using 200U/well Superscript III reverse transcriptase
773 (Thermo Fisher), 2□l of 10mM dNTP mix (Bioline, Taunton, MA) and 3□l of 150 ng/ul random
774 hexamers (Gene Link, Hawthorne, NY) under the following cycling parameters: 42°C 10 min,
775 25°C 10 min, 50°C 60 min, 94°C 5 min, and 4°C hold.

776 First strand cDNA was amplified in a 2-step multiplex nested PCR in which the first step
777 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: 95ºC 5 min; 50 cycles of 95ºC 30 sec, 58ºC
783 30 sec, 72ºC 1min; 72ºC 7 min; and 4ºC 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: 94ºC 5 min; 50 cycles of 94ºC 30 sec, *x*ºC 30 sec, 72ºC 55 sec;
789 72ºC 10 min; and 4ºC hold, where x = 54ºC for G1 mix, 48ºC for G2 mix, and 52ºC for G3 mix.
790 □-chains were amplified under the following cycling parameters: 95ºC 5 min; 50 cycles of 95ºC
791 30 sec, 50ºC 30 sec, 72ºC 1min; 72ºC 7 min; and 4ºC 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: 95ºC 5 min; 50 cycles of 95ºC 30 sec, 58ºC 30 sec, 72ºC 1min; 72ºC 7 min; and 4ºC
802 hold. □-chains were amplified under the following cycling parameters: 95ºC 5 min; 50 cycles of
803 95ºC 30 sec, 52ºC 30 sec, 72ºC 1min; 72ºC 7 min; and 4ºC hold. □-chains were amplified under
804 the following cycling parameters: 95ºC 5 min; 50 cycles of 95ºC 30 sec, 60ºC 30 sec, 72ºC 1min;
805 72ºC 7 min; and 4ºC 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

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

<u>mAb and Fab Production</u>

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

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

<u>Biotinylation of mAbs</u>

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

<u>ELISAs with Recombinant VLPs, gp120 and gp140</u>

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

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

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

881 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

898 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

(cells + virus column) and test wells (cells + serum/Ab sample + virus), dividing this result by the average RLU of virus only wells (cell + virus column) and multiplying by 100. Background is subtracted from all test wells using the average RLU from the uninfected control wells (cells only column) before calculating the percent neutralization. Neutralizing serum antibody titers are expressed as the serum dilution/antibody concentration required to achieve 50% neutralization and calculated using a dose-response curve fit with a 5-parameter nonlinear function.

<u>Neutralization Assays Using CF2 Cells</u>

Neutralization assays using CF2 cells were performed as described previously **(Crooks et al., 2015)**. On the day prior to the assay, 96 well plates were seeded with 200μl of CF2Th.CD4.CCR5 cells at $1x10^5$/ml per well. On the day of the assay, virus was incubated with serially diluted mAbs or serum for 1 hour at 37 °C. The virus:antibody/serum mixture was then added to plates seeded with CF2 cells, spinoculated at 300Xg for 15 minutes, and incubated for 3 days at 37 °C. Luciferase activity was measured using the Promega Luciferase Assay System reagent.

<u>HEp-2 Cell Staining</u>

Autoreactivity staining assays were performed on HEp-2 cells per the manufacturer recommendations (Zeus Scientific, Branchburg, NJ). MAbs were diluted to 50 and 25 μg/ml using SAVe Diluent. 20μl of the appropriate dilution was coated onto cells fixed on the slide and incubated for 30 minutes at room temperature in a humidified chamber. Slides were rinsed in 1X PBS, washed twice in 1X PBS in Coplin jars for 3-5 minutes and then stained with 20μl of FITC-conjugated secondary antibody for 30 minutes in a humidified chamber. Slides were rinsed in 1X PBS, washed twice in 1X PBS in Coplin jars for 3-5 minutes and mounted with 15μl of mounting media per well and a cover glass (Thermo Scientific). Slides were imaged on a Nikon Eclipse E800 microscope at 20X in the RGB mode for 2 seconds using SPOT 5.0 software (SPOT Imaging, Sterling Heights, MI). VRC01, 4E10, VRC07-523 and VRC07-G54W were used as control mAbs for staining and given a score of 0, 1, 2 and 3 respectively based on their staining intensity. Test antibodies were assigned scores based on visual comparisons of staining intensity to the control antibodies.

<u>Cardiolipin ELISA</u>

MAb binding to cardiolipin was tested by ELISA per the manufacturer's protocol (Inova Diagnostics, San Diego, CA). Starting at 100μg/ml, mAbs were tested in a 3-fold series. Assays

were validated using positive and negative controls and standards provided in the kit. OD values were converted to IgG anti-phospholipid (GPL) units by linear regression. 4E10 and VRC01 were used as additional positive and negative controls. Cardiolipin binding was scored as follows: no binding for <15 GPL, indeterminate for 15-20 GPL, low positive for 20-80 GPL and high positive for >80 GPL.

Genetic Clustering and Phylogenetic Analysis

MAb sequences were aligned by ClustalOmega, followed by ClustalW-Phylogeny and Dendroscope **(Huson et al., 2007)** to generate and display the phylogenetic tree. MAb lineages were inferred using *Cloanalyst* (http://www.bu.edu/computationalimmunology). Briefly, DNA Maximum Likelihood (DNAML) from the Phylip molecular evolution suite was used to find the maximum likelihood tree using the γ and κ chains together, with mutation rates differing by chain and varying by site. The inferred ancestor (IA) and intermediates were determined by computing the Bayesian posterior probability over all possible sequences representing unmutated immunoglobulin variable-region gene rearrangements.

Blue Native PAGE-Western Blot

VLPs were solubilized in 0.12% Triton X-100 in 1 mM EDTA/1.5 M aminocaproic acid with a protease inhibitor cocktail (P-2714; Sigma). An equal volume of 2X sample buffer (100 mM morpholinepropanesulfonic acid, 100 mM Tris-HCl, pH 7.7, 40% glycerol, and 0.1% Coomassie blue) was added. Samples were then loaded onto a 4-12% Bis-Tris NuPAGE gel (Thermo Fisher) and separated at 4°C for 3 hours at 100V. The gel was then blotted onto polyvinylidene difluoride and destained with a solution containing 35% methanol and 10% acetic acid followed by 100% methanol until blue stain was removed. The blot was blocked using 4% nonfat milk in PBS for 1 hour at room temperature and probed with mAbs 2G12, b12, 39F, 2F5 and 4E10 at 1µg/ml each. The blot was developed with anti-human Fc alkaline phosphatase conjugate (Jackson ImmunoResearch, West Grove, PA) diluted 1:5,000 in 2% milk in TBS-T followed by SigmaFast BCIP/NBT substrate (Sigma).

In BN-PAGE “shift” assays **(Moore et al., 2006; Tong et al., 2012)** mAbs were incubated at 37°C for 1 hour with 12.5□g gp120 equivalents of Env that was liberated from VLPs by adding 1% Triton X-100. Complexes were then resolved by BN-PAGE-Western blot as above and probed with a cocktail of biotinylated mAbs (b12, 2G12 and 4E10) followed by a streptavidin-alkaline phosphatase conjugate and BCIP/NBT.

Negative-stain EM

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

Structural Analysis

To form VRC38-1FD6-WITO-V1V2 complexes, 3mg of purified VRC38.01 HRV3C mutant IgG was bound to 750 ml Protein A Plus Agarose (Pierce) in a disposable 10ml column. 10mg of V1V2 scaffold, produced in GnTI- cells, was then added. Misfolded or improperly glycosylated scaffold remained unbound to Ab and was flushed with 5 column volumes of PBS. The column was capped and 20□l of HRV3C protease at 2U/□l was added in 1ml of PBS. After 2 hours at room temperature, the resin was drained, the eluate collected and passed over a 16/60 S200 column in buffer containing 5mM HEPES 7.5, 50mM NaCl, and 0.02% NaN_3. VRC38.01–1FD6-WITO-V1V2 complex fractions were pooled and concentrated to 10mg/ml.

Samples of monovalent VRC38.01 Fab, both unliganded and in complex with 1FD6-WITO-V1V2, were screened for crystallization using 572 conditions from Hampton, Wizard and Precipitant Synergy screens using a Cartesian Honeybee and a mosquito crystallization robot with 0.1□l of reservoir solution and 0.1□l of protein solution per condition. Crystals for VRC38.01 Fab obtained in 14% PEG400, 13% PEG8000, 0.1M Tris pH 8.5, 0.1M $MgCl_2$ were flash-frozen in liquid nitrogen with no cryoprotectant. Crystals of VRC38.01–1FD6-WITO-V1V2 obtained in 27% isopropanol, 0.1M imidazole pH 6.5 and 10% PEG8000 were flash-frozen in liquid nitrogen in mother liquor supplemented with 20% 2-methyl-2,4-pentanedial (MPD). Data were collected at 1.00Å using the SER-CAT beamline ID-22 of the Advanced Photon Source, Argonne National Laboratory (Lemont, IL).

Diffraction data were processed with the HKL2000 suite 6 (HKL Research, Inc., Charlottesville, VA). A molecular replacement solution for unliganded VRC38.01 obtained with Phenix (www.phenix-online.org) contained one Fab molecule per asymmetric unit in space group $P2_12 2_1$. The structure of the complex contained one Fab bound to a 1FD6–WITO-V1V2 monomer in space group $P3_221$. Model building was carried out using COOT software (https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/), and was refined with Phenix. Final data collection and refinement statistics are shown in supplemental Table 1. The Ramachandran plot determined by Molprobity (http://molprobity.biochem.duke.edu) shows 98.4% of all residues in favored regions and 100% of all residues in allowed regions for the unliganded Fab structure and 92.8% of all residues in favored regions and 99.2% of all residues in allowed regions for the complex structure.

Biolayer Interferometry

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

Surface Plasmon Resonance Analysis

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

Healthcare) using a 1:1 Langmuir model of binding to highlight the apparent change in affinity from Fab to IgG.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was performed using the ITC200 microcalorimeter system (MicroCal Inc., Malvern, Worcestershire, United Kingdom). Proteins were dialyzed against filtered PBS before use. The concentration of BG505 native, flexibly linked (NFL) trimer **(Sharma et al., 2015)** in the sample cell was approximately 25 µM, and that of VRC38.01 Fab in the syringe was approximately 5 µM. Reactions were carried out at 37°C, as previously described **(Wu et al., 2010)**. Briefly, BG505 NFL trimer was titrated to saturation by stepwise addition of 2 µl ligand in the syringe at 120-second intervals at 37ºC. The heat evolved upon each injection was obtained from the integral of the calorimetric signal. The values of enthalpy (ΔH) and entropy (ΔS) were obtained by fitting the data to a nonlinear least-squares analysis with Microcal ORIGIN software using a single-site binding model.

Neutralization Fingerprinting Analysis

Published neutralization data for a set of ~200 donor plasma samples **(Hraber et al., 2014)** was analyzed by using a next-generation neutralization fingerprinting algorithm **(Doria-Rose et al., 2017)**. For a given sample, the approach compares a polyclonal neutralization pattern of a set of diverse viral strains to the neutralization patterns (or fingerprints) of a reference set of broadly neutralizing mAb specificities, to obtain an estimate of the contribution of each of the reference specificities to polyclonal neutralization **(Doria-Rose et al., 2017; Georgiev et al., 2013)**. Reference broadly neutralizing antibodies were used as previously described **(Pancera et al., 2014)**, with the VRC38 fingerprint as a separate category. We applied computational quality control metrics for filtering out plasma samples for which the predictions were deemed unlikely to be accurate. Specifically, these included metrics for: (i) predicting the presence of dominant novel specificities and (ii) computing a confidence score associated with the computational predictions for each given sample **(Doria-Rose et al., 2017)**. Through this process, the initial set of samples was reduced to 80 samples, which were used for the analysis of Ab specificity frequency. Samples were predicted to have between 1 and 3 specificities from the reference set, and the overall frequency of observing the different reference specificities or pairs of reference specificities were analyzed.

QUANTIFICATION AND STATISTICAL ANALYSIS

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

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

DATA AND SOFTWARE AVAILABILITY

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

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