

HIV-1 CD4-binding site germline antibody–Env structures inform vaccine design

Received: 31 July 2022

Accepted: 4 October 2022

Published online: 17 October 2022

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BG24, a VRC01-class broadly neutralizing antibody (bNAb) against HIV-1 Env with relatively few somatic hypermutations (SHMs), represents a promising target for vaccine strategies to elicit CD4-binding site (CD4bs) bNAbs. To understand how SHMs correlate with BG24 neutralization of HIV-1, we report 4.1 Å and 3.4 Å single-particle cryo-EM structures of two inferred germline (iGL) BG24 precursors complexed with engineered Env-based immunogens lacking CD4bs N-glycans. Structures reveal critical Env contacts by BG24_{iGL} and identify antibody light chain structural features that impede Env recognition. In addition, biochemical data and cryo-EM structures of BG24_{iGL} variants bound to Envs with CD4bs glycans present provide insights into N-glycan accommodation, including structural modes of light chain adaptations in the presence of the N276_{gp120} glycan. Together, these findings reveal Env regions critical for germline antibody recognition and potential sites to alter in immunogen design.

Current strategies to engineer a vaccine towards preventing HIV-1 infection involve designing Env-mimetic immunogens that can elicit broadly neutralizing antibodies (bNAbs)^{1–4}. The CD4-binding site (CD4bs) epitope is a target of immunogen design as bNAbs in this class have been shown to be among the most potent and broad^{5–9}. Several studies have shown passive immunization using CD4bs bNAbs can confer protection from HIV-1 infection in animal models and human clinical trials, suggesting that immunization strategies to elicit these antibodies at effective concentrations would also be protective^{6,10–17}. This includes the VRC01-class of bNAbs that are derived from the VH1-2*02 variable heavy chain gene segment and are characterized by a short 5 amino acid complementary determining region 3 (CDR3) in the antibody (Ab) light chain and a shortened or flexible CDRL1^{5,18}. These characteristics are necessary for VRC01-class bNAbs to accommodate the heavily N-glycosylated landscape of the CD4bs of HIV-1 Envs. Thus,

VRC01-class bNAbs generally require high levels of somatic hypermutation (SHM), which is challenging to elicit through vaccination.

Germline precursors of bNAbs do not generally show detectable binding to non-engineered, natively-glycosylated HIV-1 Envs^{19,20}, therefore, the germline-targeting approach to HIV-1 vaccine design involves efforts to engineer immunogens that can engage germline B-cell receptors (BCRs) and initiate bNAb development²¹. Inferred germline (iGL) versions of mature bNAbs derived from predicted germline gene segment sequences represented in the human B-cell repertoire^{22,23} are used for the germline-targeting approach. Analysis of VRC01-class iGLs has shown that the human VH1-2*02 heavy chain gene segment encodes signature residues that are required for breadth and potency¹⁸. Furthermore, germline VRC01-class precursors have been isolated from naïve individuals, and mature bNAbs have been identified from multiple HIV-1-infected human donors, suggesting that

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raising this class of bNAbs is not uncommon in natural infection^{24,25}. Taken together, VRC01-class bNAbs are attractive targets for immunogen design.

The VRC01-class of bNAbs targets a particularly challenging epitope to elicit bNAbs against due to the presence of the CD4bs N-glycans that sterically obstruct interactions between Env and Ab CDRs²⁶. The glycan at position N276_{gp120} is highly conserved and poses the greatest steric barrier to binding VRC01-class bNAb iGLs, as Ab residues in the iGL CDRL1 that interact with this region are typically 11–12 residues and cannot accommodate the N276_{gp120} glycan. Mature CD4bs Abs develop shortened or flexible CDRL1s to accommodate this glycan^{24,27,28}. Thus, understanding the structural basis for how CD4bs iGL Abs mature to effectively accommodate the N276_{gp120} glycan is essential in efforts to develop effective immunogens to prime VRC01-class iGL precursors and shepherd antibody responses towards bNAb development. Furthermore, an overall structural understanding of VRC01-class iGL recognition of HIV-1 Envs and immunogens is limited as the only existing Fab-Env structures involving germline CD4bs Abs are complexed with gp120 or Env trimer immunogens lacking the N276_{gp120} glycan^{3,23,29}. In addition, in the case of an iGL Fab complexed with an Env trimer, obtaining a structure required chemical cross-linking between the Env and Ab to form a stable complex²².

A VRC01-class bNAb isolated from an elite neutralizer, BG24³⁰, is an attractive target for germline-targeting immunogen design. BG24 shows similar neutralization and breadth to other CD4bs bNAbs, but includes only 22.6% and 19.5% amino acid substitution by SHM in variable heavy and light chain genes, respectively³⁰, as compared with higher levels of amino acid substitution in VRC01-class bNAbs^{7,9,28,31}, with the exception of the PCIN63 lineage that has similar levels of SHM to BG24³². Structural characterization of BG24 bound to the clade A BG505 Env revealed a similar binding orientation to more mutated VRC01-class bNAbs, and signature contacts common to VRC01-class bNAbs³⁰. Furthermore, neutralization studies using variants of BG24 that reverted variable heavy (V_H) and variable light (V_L) domain residues to germline counterparts showed that even fewer SHMs were necessary to maintain neutralization breadth³⁰. Collectively, this suggests broad and potent neutralization targeting the CD4bs could be achieved through immunization without stimulating high levels of SHM.

In this work, we structurally characterize the binding of two versions of the BG24 iGL to the CD4bs germline-targeting immunogen BG505-SOSIPv4.1-GT1³ (hereafter referred to as GT1), to better understand how the BG24 bNAb was elicited and inform VRC01-class immunogen design. We solve two single-particle cryo-electron microscopy (cryo-EM) structures of GT1 in complex with BG24_{iGLs} containing either mature or iGL CDR3s at 4.1 and 3.4 Å resolution, respectively, in both cases in the absence of chemical crosslinking. Furthermore, to understand how N-glycans impact germline Ab recognition of Env, we conduct biochemical assays and solve cryo-EM structures of BG24_{iGL} derivatives bound to Envs that included the N276_{gp120} glycan. The structures demonstrate that the CDRL1s of BG24_{iGLs} can adopt conformations that accommodate the N276_{gp120} glycan, an important capability for a germline-targeting CD4bs immunogen. Collectively, these structures provide information regarding the physical characteristics of iGLs that recognize HIV-1 Env and provide a structural basis for the design of immunogens engineered to engage and mature germline Abs.

Results

Cryo-EM structures of GT1-BG24_{iGL}-10-1074 complexes

To gain insight into how BG24 precursors interact with an HIV-1 Env-based immunogen, we created iGL versions of BG24 and used single-particle cryo-EM to structurally characterize them in complex with GT1, a CD4bs germline-targeting immunogen³. GT1 was modified

from a soluble clade A BG505 SOSIP.664 native-like Env trimer³³ to permit binding of VRC01-class germline precursors by including T278R_{gp120} and G471S_{gp120} substitutions and mutations to remove potential N-linked glycosylation sites (PNGSs) at positions N276_{gp120}, N462_{gp120}, N386_{gp120}, and N197_{gp120} in the CD4bs³. Two iGL versions of BG24 Fab constructs were made starting with the VHI-2*02 and VL2-11*01 heavy and light chain germline gene segment sequences: one containing the CDR3s from mature BG24 (BG24_{iGL-CDR3mat}) and the other containing the iGL CDR3s (BG24_{iGL-CDR3iGL}) (Fig. 1a). Each BG24_{iGL} was structurally characterized in complex with GT1 and the V3 bNAb 10-1074³⁴.

Cryo-EM structures of BG24_{iGL-CDR3iGL} and BG24_{iGL-CDR3mat} Fabs bound to GT1 were solved at 3.4 and 4.1 Å, respectively (Fig. 1b, c, Supplementary Fig. 1a–j, Supplementary Table 1). Both 3D cryo-EM reconstructions showed three BG24_{iGL} and three 10-1074 Fabs bound per Env trimer. However, for the BG24_{iGL-CDR3iGL}-GT1-10-1074 complex, a distinct 3D class contained two BG24_{iGL-CDR3iGL} Fabs bound to the GT1 Env (Supplementary Fig. 1e, f, Supplementary Table 1). We also solved a 1.4 Å crystal structure of unbound BG24_{iGL-CDR3mat} Fab (Supplementary Fig. 1k, Supplementary Table 2), which exhibited six disordered residues within CDRL1, but otherwise superimposed with a 1.3 Å root mean square deviation (rmsd; calculated for 225 V_H-V_L Cα atoms) with the Env-bound BG24_{iGL-CDR3mat} Fab structure, suggesting no major structural differences upon Env binding.

BG24_{iGL} Fabs recognize the modified CD4bs in GT1 Env

The GT1 complexes with BG24_{iGLs} included density for CD4bs N-glycans attached to residues N234_{gp120}, N363_{gp120}, and N392_{gp120} (Fig. 2a, b). These N-glycans were also observed in the crystal structure of BG505 Env complexed with a mature BG24 Fab³⁰ (BG24_{mat}) (PDB 7UCF), which also included densities for N-glycans at N197_{gp120}, N276_{gp120}, and N386_{gp120} that are not present in GT1 (Fig. 2c). Despite additional glycans in BG505 compared with GT1, the CDR loops in the GT1-bound iGL Fabs showed similar orientations and positions as in the BG505-bound BG24_{mat} Fab, except for CDRL1, which is six residues longer in BG24_{iGL} than in BG24_{mat} (Figs. 1a, 2d–f).

BG24_{iGL-CDR3mat} and BG24_{iGL-CDR3iGL} buried comparable surface areas on GT1 gp120 (953 and 951 Å², respectively) as compared with only slightly larger surface area (1086 Å²) buried on BG505 gp120 in the BG24_{mat}-BG505 structure (PDB 7UCF) (Fig. 2g). We hypothesize that, although a germline precursor antibody presumably exhibits fewer contacts to an antigen than its counterpart somatically mutated bNAb, the overall interface BSA values for the gp120 peptide components of the BG24_{iGL}-GT1 and BG24_{mat}-BG505 structures were similar because the modifications in GT1 (both amino acid substitutions and removals of N-glycans) allowed increased contacts between BG24_{iGLs} and the GT1 gp120. However, differences in interactions between the BG24_{iGL}-GT1 and BG24_{mat}-BG505 structures suggest that SHM substitutions enrich interactions in particular regions within the CD4bs (Fig. 2g, h). For example, in the BG24_{mat}-BG505 complex, BG24_{mat} residue S100A_{HC} hydrogen bonds with the gp120 inner domain residue K97_{gp120} (Fig. 2g–j). K97_{gp120} is ~90% conserved among HIV-1 Envs, making this a crucial interaction of broad and potent CD4bs bNAbs¹⁸. Residue S100A_{HC} is a germline-encoded residue, however, in both BG24_{iGL}-GT1 structures, is not within the distance to form a hydrogen bond with K97_{gp120} (Fig. 2l, j). Compared to BG24_{iGL}-GT1, BG24_{mat}-BG505 also showed increased V_H buried surface area (BSA) in the gp120 exit loop (gp120 residues 472–476). Together, this analysis demonstrated differences in the distribution of BSA among BG24_{mat} and BG24_{iGLs} in CD4bs regions.

BG24 somatic hypermutation and germline features play a role in CD4bs recognition

We next compared how differences in BG24_{iGL} and BG24_{mat} contribute to their recognition of GT1 and BG505, respectively. BG24_{iGL} contains a

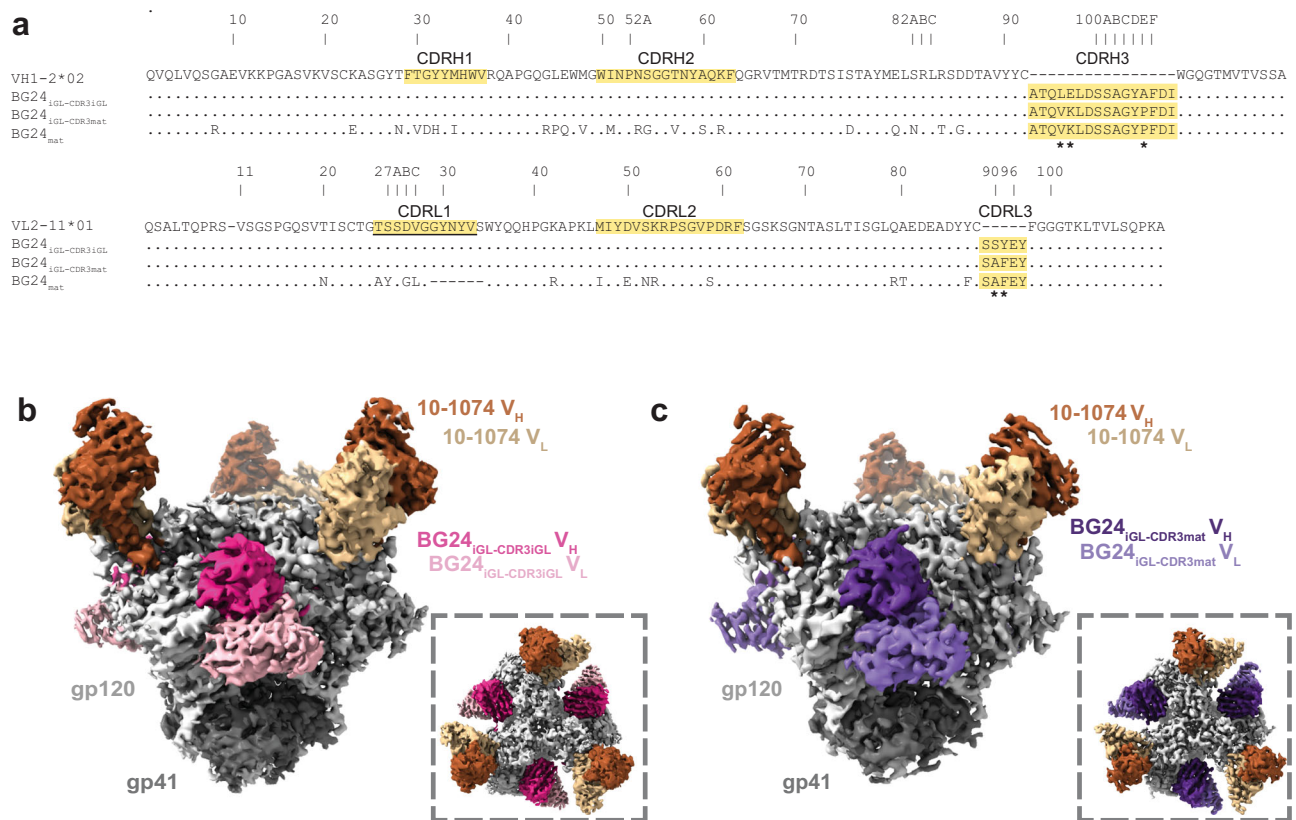


Fig. 1 BG24_{iGL}s bind the CD4bs of the GT1 immunogen. **a** Sequence alignment of V_H and V_L iGL precursors of BG24 (VH1-2*02 and VL2-11*01), BG24_{iGL-CDR3iGL}, BG24_{iGL-CDR3mat}, and BG24_{mat}. CDRs are highlighted in yellow. Asterisks (*) indicate residue differences between mature and iGL CDR3s. Underlined CDRL1 indicates sequence used for the CDRL1 in the BG24_{CDRL1-iGL} construct. **b, c** Side and top-down (inset) views of cryo-EM density of BG24_{iGL-CDR3iGL}-GT1-10-1074 (**b**) and BG24_{iGL-CDR3mat}-GT1-10-1074 (**c**). Highlighted in colors include: gp120 subunits (light gray), gp41 (dark gray), BG24_{iGL-CDR3iGL} V_H (bright pink) and V_L (light pink) domains, BG24_{iGL-CDR3mat} V_H (dark purple) and V_L (light purple) domains, and 10-1074 V_H (dark brown) and V_L (light brown) domains.

germline I1-residue CDRL1 that can recognize the mostly aglycosylated CD4bs in GT1, whereas the BG24_{mat} CDRL1 is six residues shorter and includes a glycine to create a more flexible loop that can accommodate the N276_{gp120} glycan³⁰. In the BG24_{mat}-BG505 structure, the five-residue BG24 CDRL1 is oriented adjacent to the N276_{gp120} glycan (Fig. 3a). The CDRL1 interface with GT1 in the BG24_{iGL-CDR3iGL} and BG24_{iGL-CDR3mat} structures showed the longer CDRL1s in the germline precursor V_L domains in different conformations, demonstrating CDRL1 flexibility (Fig. 3b, c) consistent with cryo-EM data processing. Indeed, the local resolutions for the CDRL1 in these structures were poor and resolved only after iterative rounds of focused classification and local refinements (Supplementary Fig. 2a, b). Overlaying the BG24_{iGL} CDRL1s with the gp120 region surrounding the N276_{gp120} glycan from the BG24_{mat}-BG505 structure showed steric clashes, consistent with SHM being necessary for N276_{gp120} glycan accommodation by BG24 (Fig. 3b, c).

The role of SHMs in Env recognition is summarized in Fig. 3d, where BG24_{iGL-CDR3iGL}, BG24_{iGL-CDR3mat}, and BG24_{mat} HC paratope interactions are mapped to individual Ab residues within 4 Å of gp120. Paratope contacts were limited to CDR H1, H2, and H3, as well as framework region 3 in the heavy chain (FWRH3), with most contacts in CDRH2. Previous studies showed neutralization by an engineered BG24 minimal construct that contained germline-reverted SHMs in FWRs, CDRH1, and CDRL2, but maintained most SHMs in CDRH2, suggesting the importance of SHMs in this region³⁰. The structure of BG24_{mat}-BG505 showed a CDRH2 SHM (N53R_{HC}) interacted with Q428_{gp120} in gp120 β20/21 (Figs. 1a, 3e). β20/21 interactions with germline-encoded N53_{HC} were absent in BG24_{iGL-CDR3iGL}-GT1 and BG24_{iGL-CDR3mat}-GT1 (Fig. 3f, g). This demonstrates the direct impact of

SHM in creating favorable interactions with Env. Other BG24_{mat} somatically hypermutated residues in CDRH2 also interacted with the CD4bs loop (gp120 residues 364–375); e.g., residue T57V_{HC} makes a backbone interaction with S365_{gp120}, and S54_{HC} interacts with D368_{gp120}, a highly conserved Env residue (Fig. 3d)¹⁸. For BG24_{iGL}, germline-encoded residues at positions T57_{HC} and S54_{HC}, maintain similar interactions with GT1 residues S365_{gp120} and D368_{gp120}, respectively (Fig. 3f, g).

To understand the functional role of these CDRH2 SHMs, we tested the neutralization activity of BG24_{mat} constructs in which individual SHMs were reverted to the corresponding germline residue (BG24 R53N_{HC}, BG24 G54S_{HC}, and BG24 V57T_{HC}) and a combined construct with all three mutations reverted (BG24 CDRH2_{iGL-3mut}) against a 12-strain global panel of HIV-1 strains³⁵ plus BG505 T332N³³, and compared potencies and breadth against BG24_{mat} (Fig. 3h). For BG24 constructs with R53N_{HC} and V57T_{HC} single mutations, we observed a greater than 5-fold decrease in potency against 6 of the 13 strains tested compared to BG24_{mat}. BG24 G54S_{HC} most closely compared to BG24_{mat}, showing a more than five-fold decrease in potency against only 3 strains. BG24 CDRH2_{iGL-3mut} was more strongly affected by the SHM reversions, showing more than a five-fold decrease in potency against almost all strains compared the BG24_{mat}. These results demonstrate that SHMs in CDRH2 shown to interact favorably with Env also play a functional role by contributing to neutralization potency and breadth.

Signature residues encoded by the VH1-2*02 germline gene in VRC01-class bNAbs interact with conserved gp120 residues and are correlated with neutralization potency¹⁸. These interactions have been structurally characterized in the context of VRC01-class iGLs bound to

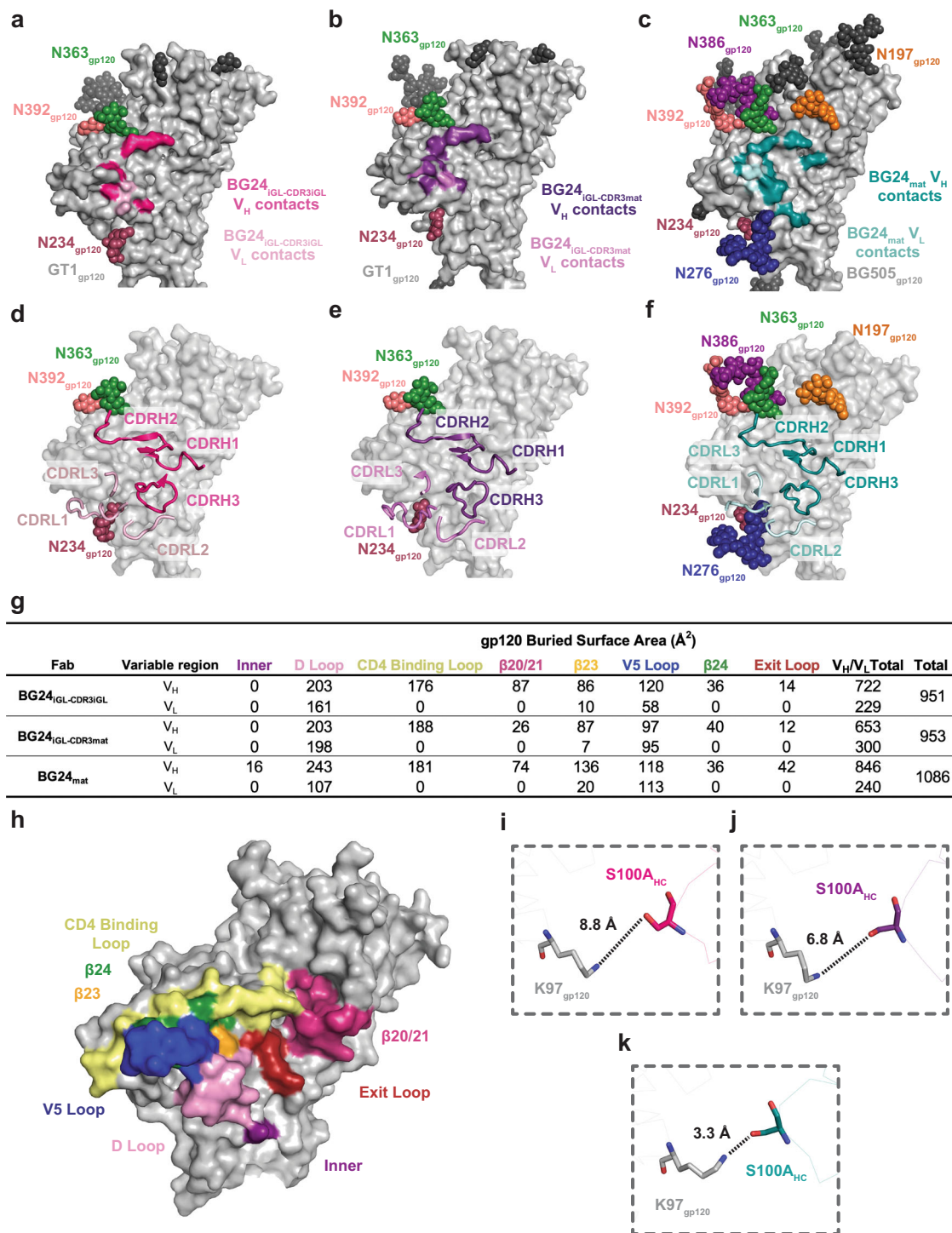


Fig. 2 | Comparison of BG24_{I_{GL}} and BG24_{mat} CD4bs epitopes. Surface contacts made by BG24_{I_{GL}-CDR3_{IGL} V_H (bright pink) and V_L (light pink) on GT1 gp120 (light gray) (a), BG24_{I_{GL}-CDR3_{mat} V_H (dark purple) and V_L (light purple) on GT1 gp120 (light gray) (b), and BG24_{mat} V_H (deep teal) and V_L (light teal) on BG505 gp120 (light gray) (PDB 7UCF). (c) Surface representation of gp120 (light gray) with cartoon representations of BG24_{I_{GL}-CDR3_{IGL} (d), BG24_{I_{GL}-CDR3_{mat} (e), and BG24_{mat} (f) CDR loops. V_H and V_L CDR loops are colored the same as in a–c. **g** Summary table of gp120 buried surface area (BSA) (Å²) calculations for BG24_{I_{GL}-CDR3_{IGL}, BG24_{I_{GL}-CDR3_{mat}, and BG24_{mat} at the inner domain (inner), D loop, CD4bs loop, β20/21, β23,}}}}}}

V5 loop, β24, and exit loop of the CD4bs. BSA calculations were conducted for gp120 peptide components and did not include glycan interactions. **h** Surface representation of gp120 (PDB 5T3Z) with CD4bs motifs colored and labeled (inner domain—purple, D loop—light pink, CD4bs loop—yellow, β20/21—bright pink, β23—orange, V5 loop—blue, β24—green, and exit loop—red). Distance measurements between K97_{gp120} and S100A_{HC} for **i** BG24_{I_{GL}-CDR3_{IGL}-GT1_{gp120}, **j** BG24_{I_{GL}-CDR3_{mat}-GT1_{gp120}, and **k** BG24_{mat}-BG505_{gp120} structures. The distances between atoms are represented by black dotted lines.}}

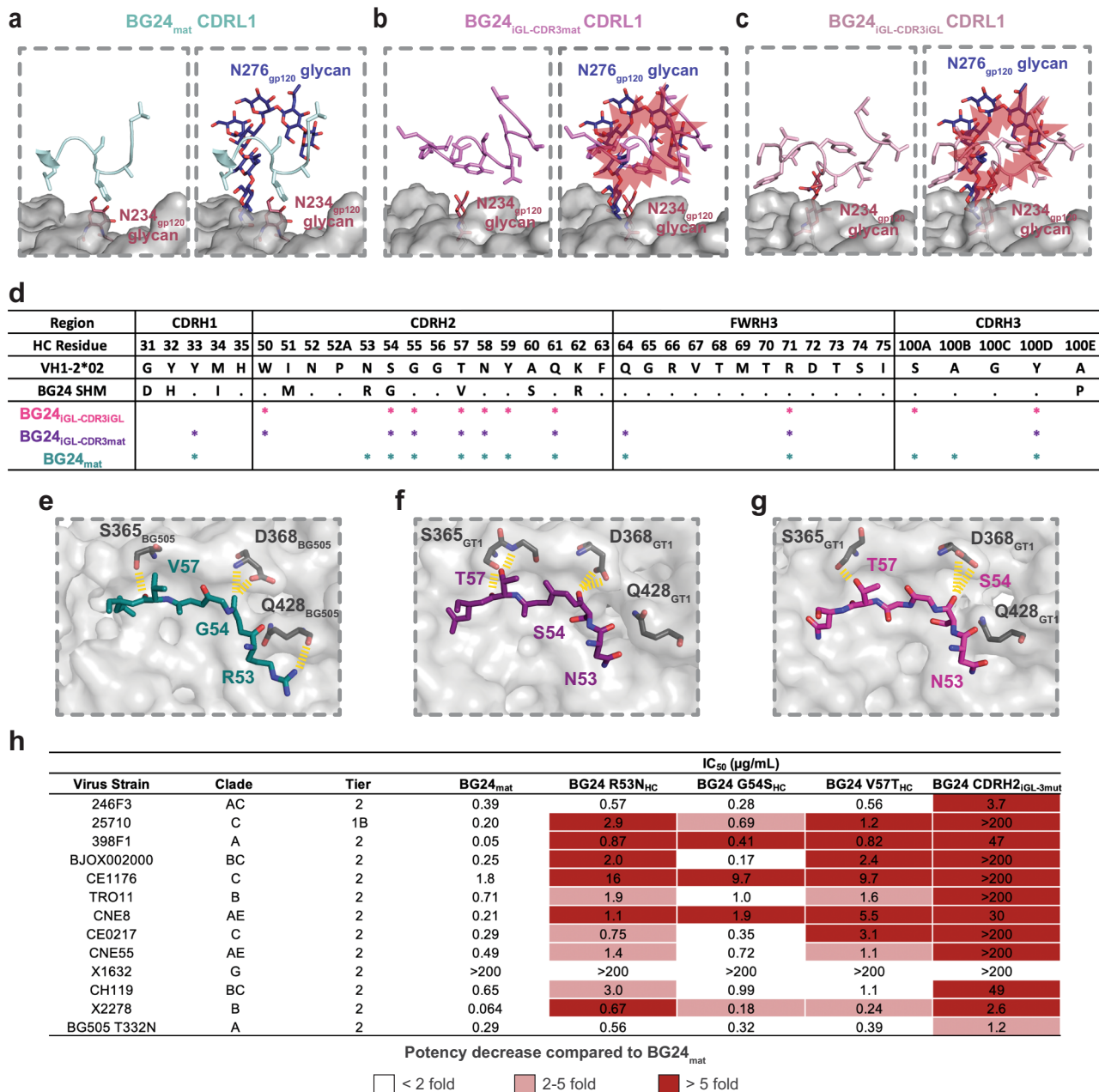


Fig. 3 | Somatic hypermutation and germline features play a role in BG24 recognition of the CD4bs interface. gp120 (light gray) surface in the vicinity of the CD4bs with cartoon representation main chain/stick side chains for the CDR1s of **a** BG24_{mat} (light teal), **b** BG24_{I_{GL}-CDR3_{mat}} (light purple), and **c** BG24_{I_{GL}-CDR3_{I_{GL}}} (light pink) overlaid with the N276_{gp120} N-glycan (dark blue) from the BG24_{mat}-BG505 complex (PDB 7UCF). Steric clashes are represented with red bursts. **d** Table summarizing HC paratope residues in BG24_{I_{GL}-CDR3_{I_{GL}}}-GT1, BG24_{I_{GL}-CDR3_{mat}}-GT1, and BG24_{mat}-BG505 structures. The paratope was defined by Ab residues that make

contacts with gp120 within 4 Å for each structure. Stick representations of the CDRH2 residues from **e** BG24_{mat} (deep teal), **f** BG24_{I_{GL}-CDR3_{mat}} (dark purple), and **g** BG24_{I_{GL}-CDR3_{I_{GL}}} (bright pink) interacting with BG505 or GT1 gp120 residues. Yellow dashed lines indicate Ab-gp120 interactions within 4 Å. **h** Neutralization data of BG24 CDRH2 mutants against a global 12 virus panel³⁵ and BG505 T332N³³. IC₅₀ values represent the average mean from duplicate neutralization measurements. A heatmap describes <2-fold (white), 2–5-fold (light red), and >5-fold (dark red) potency decreases compared to BG24_{mat}.

monomeric gp120s^{22,23,29}, but there are no known structures of VRC01-class iGLs bound to a trimeric Env, except when the iGL was chemically cross-linked to Env²². To evaluate and verify VRC01-class VH1-2*02 germline-encoded interactions with an Env trimer, we compared these interactions in the BG24_{I_{GL}-GT1} and BG24_{mat}-BG505 structures (Supplementary Fig. 2c–f). Specifically, as previously described in structures involving gp120 monomers^{22,23,29}, germline-encoded R71_{HC} in the BG24_{I_{GL}-GT1} and BG24_{mat}-BG505 structures formed a salt bridge with the conserved D368_{gp120} side chain, an Ab interaction that mimics the interaction of host receptor residue R59_{CD4} with D368_{gp120}

(Supplementary Fig. 2c). In the gp120 D loop, there were interactions between the backbone and side chain of N280_{gp120} with Y100D_{HC} and germline-encoded W50_{HC} side chains (Supplementary Fig. 2d). In the V5 loop, interactions between the conserved R456_{gp120} residue and germline-encoded N58_{HC} are conserved in both structures (Supplementary Fig. 2e). In BG24_{I_{GL}-CDR3_{mat}}-GT1, atoms within these Fab residues were separated by more than 5 Å from atoms within gp120 residues; thus, this is not defined as an interaction. In the light chain, E96_{LC} interacted with the backbone of G459_{gp120} and the side chain of N280_{gp120} (Supplementary Fig. 2f).

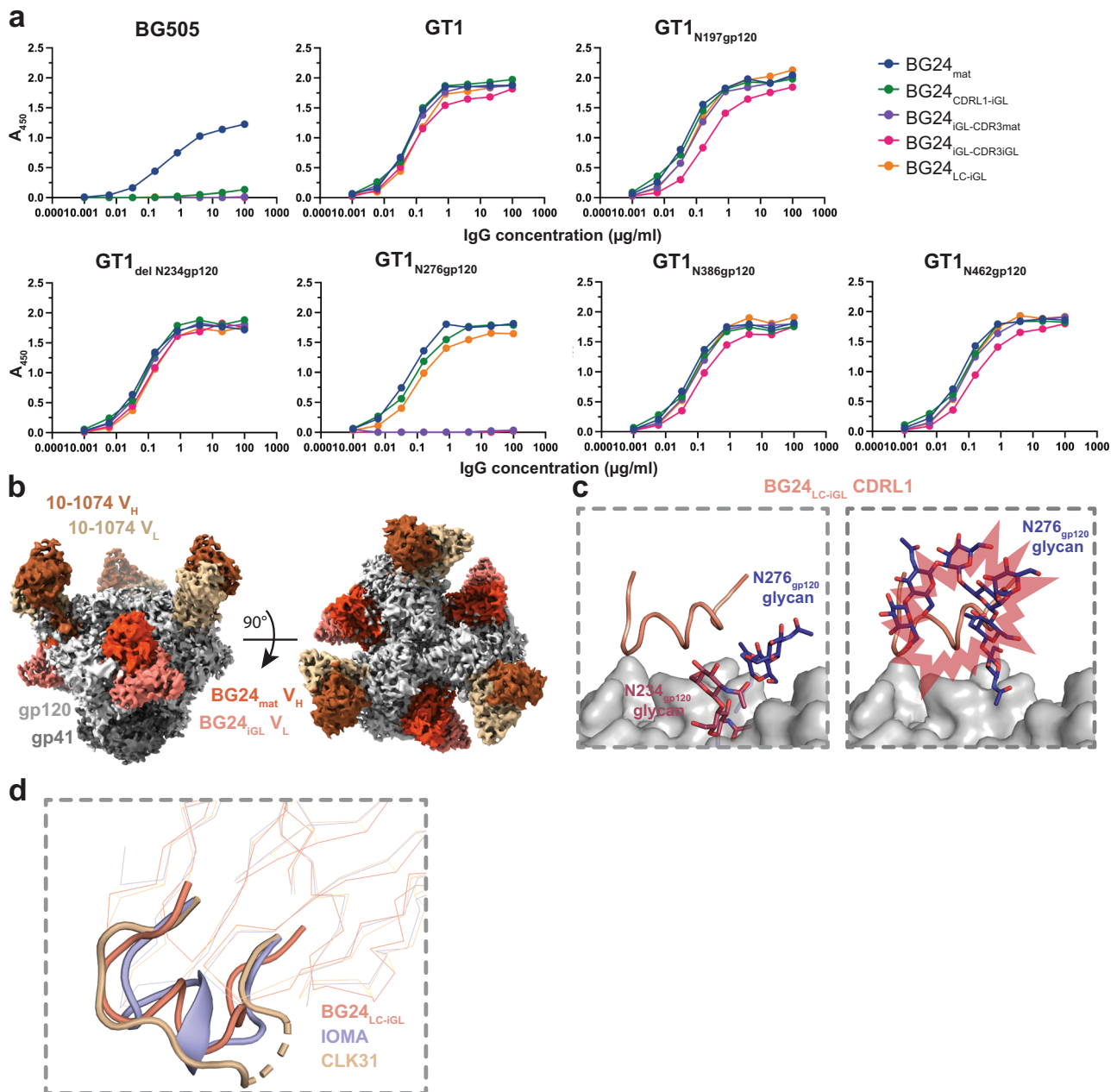


Fig. 4 | BG24_{iGL} binding is mediated by CD4bs glycans. **a** ELISA to access binding of the indicated BG24 Abs to BG505, GT1, and GT1 SOSIP Envs with altered N-glycans in the CD4bs. Streptavidin plates were coated with randomly biotinylated SOSIPs and incubated with IgGs at increasing concentrations. Values are shown as mean of two individual biological replicates ($n = 2$). Source data are provided as a Source Data file. **b** Side and top-down views of cryo-EM density of BG24_{LC-iGL}-GT1_{N276gp120}-10-1074. Highlighted in colors include: gp120 subunits

(light gray), gp41 (dark gray), BG24_{mat} V_H (deep coral), and V_L (light coral) domains, and 10-1074 V_H (dark brown) and V_L (light brown) domains. **c** Cartoon representation of the CDRL1 of BG24_{LC-iGL} (left) (light coral) and overlaid with the N276_{gp120} N-glycan (dark blue) from a BG24_{mat}-BG505 (PDB 7UCF) (right). Predicted steric clashes are indicated by red bursts. **d** Alignment of BG24_{LC-iGL} (from the BG24_{LC-iGL}-GT1_{N276gp120}-10-1074 structure) (light coral), IOMA (PDB 5T3Z) (lilac), and CLK31 (PDB 6D2P) (wheat) LC with CDRL1s represented in cartoon.

GT1 CD4bs glycan modifications affect BG24 binding

To evaluate how glycan modifications in the GT1 immunogen contributed to BG24_{iGL} binding, we evaluated the binding of BG24 constructs to GT1 with Env PNGSs either restored to or removed from the CD4bs (Fig. 4a). The BG24 constructs included BG24_{mat}, BG24 with germline CDRL1 (BG24_{CDRL1-iGL}) (Fig. 1a), BG24_{iGL-CDR3mat}, BG24_{iGL-CDR3iGL}, and BG24 with an iGL light chain (BG24_{LC-iGL}). PNGSs were individually restored at positions N197_{gp120}, N276_{gp120}, N386_{gp120}, and N462_{gp120} and removed at N234_{gp120} to create five GT1 constructs with altered glycan landscapes (GT1_{N197gp120}, GT1_{N276gp120}, GT1_{N386gp120}, GT1_{N462gp120}, and GT1_{del N234gp120}, respectively). BG505

and GT1 binding was evaluated by enzyme-linked immunosorbent assays (ELISAs). Restoring Env PNGSs at positions N197_{gp120}, N386_{gp120}, and N462_{gp120} and removing the PNGS at N234_{gp120} did not greatly affect the binding of BG24 IgG constructs (Fig. 4a). BG24_{iGL} constructs did not bind detectably to GT1 with a PNGS at N276_{gp120}; however, BG24_{mat} and mature BG24 constructs with iGL LC features (BG24_{CDRL1-iGL}, BG24_{LC-iGL}) showed comparable binding to each other on GT1_{N276gp120} (Fig. 4a). BG24_{mat} was the only Ab that showed substantial binding to BG505, which unlike the GT1 Env, included all PNGSs. We conclude that BG24 constructs with a long, germline CDRL1 can accommodate the N276_{gp120} glycan on Envs that

have been engineered to have a limited glycan landscape in the CD4bs. These results contribute to existing studies of N276_{gp120} glycan accommodation by germline CDRL1 (e.g., ref. 36).

To gain insight into BG24 CDRL1-iGL interactions with GT1 containing an N276_{gp120} glycan, we solved a single-particle cryo-EM structure of BG24_{LC-iGL} bound to GT1 containing the restored N276_{gp120} PNGS (GT1_{N276gp120}) (Fig. 4b, Supplementary Fig. 3a–h, Supplementary Table 1). We identified three unique 3D volumes containing either one, two, or three bound BG24_{LC-iGL} Fabs, with the highest resolution complex (4.2 Å) being C3 symmetric with three bound BG24_{LC-iGL} Fabs (Supplementary Fig. 3a–h, Supplementary Table 1). Electron density in the Fab CDRL1 was not optimal after cryo-EM processing; therefore, side chains were not modeled (Supplementary Fig. 3i).

The BG24_{LC-iGL}-GT1_{N276gp120} complex structure showed that the Fab CDRL1 main chain residues adopted a helix-like conformation to accommodate the N276_{gp120} glycan (Fig. 4c). Available crystallographic and cryo-EM Env structures demonstrate that the N276_{gp120} glycan is conformationally heterogeneous^{6,27,30,37,38} (Supplementary Fig. 3h). Indeed, the N276_{gp120} glycans in the GT1 and BG505 Envs exhibited different conformations (Fig. 4c). Thus, after superimposing the gp120 residues in the BG24_{LC-iGL}-GT1_{N276gp120} and BG24_{mat}-BG505 structures, it was evident that the N276_{gp120} glycan in BG505 showed steric clashes with the CDRL1-iGL in BG24_{LC-iGL} (Fig. 4c). Flexibility of the N276_{gp120} glycan on BG505 may be more constrained than the counterpart glycan on GT1, as GT1 contains fewer N-glycans in the CD4bs, allowing for increased N276_{gp120} glycan flexibility. This assumption is consistent with the ELISA results showing that BG24_{LC-iGL} bound to GT1, but not to BG505 Env trimers with an N276_{gp120} glycan (Fig. 4a).

The only other known CD4bs-targeting bNAb with a helical CDRL1 is IOMA, another VHI-2*02-derived bNAb²⁷. IOMA contains features that distinguish it from VRC01-class bNAbs, including a normal length (8-residue) CDRL3 and a 13-residue CDRL1, which adopts a short α -helix to accommodate the glycan at N276_{gp120}. However, CLK31, an IOMA-like Ab isolated from naive human B cells using a VRC01 germline-targeting immunogen, did not include a helical CDRL1²². Alignment of the LCs of BG24_{LC-iGL}, IOMA, and CLK31 showed that each CDRL1 adopts a different configuration (Fig. 4d). These observations suggest CDRL1 helical conformations are diverse and have only been observed when bound to gp120s that contain the glycan at N276_{gp120}.

BG24_{CDRL1-iGL} accommodates the N276_{gp120} glycan in a non-engineered Env trimer

A longitudinal study that tracked the development of a VRC01-class lineage (PCIN63) found that bNAb development branched into two types of N276_{gp120} glycan engagement: one that interacted with and depended on the presence of the N276_{gp120} glycan, and one in which CD4bs binding was diminished by the presence of the N276_{gp120} glycan³². In the absence of longitudinal data for BG24_{mat} development, an engineered BG24 intermediate, BG24_{CDRL1-iGL}, was tested in previous work for neutralization against a 119-virus cross-clade panel to better understand how the germline BG24 CDRL1 interacted with HIV-1 Envs bearing the N276_{gp120} glycan³⁰. BG24_{CDRL1-iGL} exhibited neutralization activity against two viruses that contained PNGSs at N276_{gp120}: clade D 6405.v4.c34 (6405) and clade CD 6952.v1.c20 (6952) (Fig. 5a)³⁰. The 6405 Env was selected for further investigation by creating a soluble 6405 SOSIP.664 trimer. Sequence alignment of 6405 and BG505 gp120s showed the amino acid identity in the CD4bs and V4 loops differed by more than 50% between BG505 and 6405 Envs (Supplementary Fig. 4). The 6405 gp120 sequence included similar CD4bs PNGSs as BG505, except for the absence of a PNGS at position 363_{gp120} and an added PNGS at position 465_{gp120} (Supplementary Fig. 4). We note that the degree to which each PNGS is utilized, the chemical and structural heterogeneity of N-glycans that are attached,

and potential cell line and virus versus soluble Env trimer differences in glycosylation are not known for 6405. The binding of BG24 Fab constructs to 6405 was assessed by ELISA. Consistent with neutralization results (Fig. 5a), ELISAs showed that BG24_{CDRL1-iGL} and BG24_{mat} each bound the 6405 SOSIP, whereas BG24_{LC-iGL} bound 6405 to a lesser extent (Fig. 5b). BG24_{iGLs} did not bind detectably to 6405 (Fig. 5b).

To understand how the germline CDRL1 of BG24 could be accommodated by a non-engineered Env trimer, we characterized interactions between BG24_{CDRL1-iGL} and 6405 SOSIP by solving a 3.4 Å cryo-EM structure of a BG24_{CDRL1-iGL}-6405 complex (Fig. 5c, Supplementary Fig. 5a–d, Supplementary Table 1). As expected, BG24_{CDRL1-iGL} recognized the CD4bs of 6405, which contained N-glycans at positions N197_{gp120}, N234_{gp120}, N276_{gp120}, N354_{gp120}, N386_{gp120}, N392_{gp120}, N461_{gp120}, and N465_{gp120} (Fig. 5d). Again, side chains were not modeled for CDRL1 residues (Supplementary Fig. 5e). As also observed in the BG24_{LC-iGL}-GT1_{N276} structure, the CDRL1-iGL in the BG24_{CDRL1-iGL}-6405 complex formed a helical conformation, although the CDRL1 conformations in the two Fabs in these complexes were not identical (Supplementary Fig. 5f). By aligning gp120s from GT1_{N276} and 6405, we found that the well-ordered portions of the N276_{gp120} glycan occupied similar positions (Fig. 5f), suggesting that the position of N276_{gp120} in these Envs is conducive to CDRL1 accommodation for BG24_{iGLs}.

We also evaluated the binding of other CD4bs bNAbs to 6405 and a 6405_{delN276gp120} Env to determine if the CD4bs glycan landscape in 6405 was conducive to interactions with germline CDRL1s in other CD4bs bNAbs (Fig. 5g, h, Supplementary Fig. 6). In this experiment, we included mature versions of the BG24, VRC01, N6 and IOMA bNAb Fabs, chimeric bNAb Fabs including an iGL LC, and complete iGL Fabs. PCIN63 and CHI03 and intermediates were compared with their unmutated common ancestors (UCAs), as identified from longitudinal studies^{32,39}, instead of iGLs. The ELISA revealed that the 6405 Env interacted with VRC01_{LC-iGL} and N6_{LC-iGL} in addition to BG24_{LC-iGL}. Binding for all species increased for 6405_{delN276gp120}, indicating the N276_{gp120} glycan can sterically impede the CDRL1 of the iGLs tested. We conclude that the 6405 Env tolerates the binding of some germline VRC01-class CDRL1s.

Discussion

VRC01-class bNAbs are promising targets for germline-targeting immunogen design as germline-encoded residues make signature contacts with gp120 that contribute to the impressive breadth and potency^{5,18,22,23}. However, challenges in eliciting VRC01-class bNAbs through a germline-targeting vaccine regimen include explicitly selecting for the VHI-2*02 germline gene, overcoming CD4bs glycan barriers, and stimulating high levels of SHM^{40,41}. Despite these challenges, progress has been made in developing a VRC01-class bNAb germline-targeting approach^{21,41,42}, which is initiated by engineering an immunogen that binds to the germline precursors of VRC01 bNAbs. Priming immunogens are engineered to interact with specific germline-encoded residues and lack CD4bs glycans that obstruct germline recognition^{3,29,40,43}. VRC01-class priming immunogens include monomeric gp120 cores^{25,40,44}, SOSIP-based trimers³, and anti-idiotypic antibodies that recognize target BCRs with VHI-2*02 gene segments^{45,46}. Furthermore, selecting a particular strain of Env and a gp120- versus trimeric Env-based platform to engineer priming characteristics that have proven to impact germline BCR selection in vivo⁴⁷. Thus, identifying and developing the optimal priming immunogen for VRC01-like bNAb elicitation will require a robust understanding of the structural and biophysical nature of Env recognition by germline precursors.

In a sequential immunization approach, boosting immunogens are introduced to shape the development of a germline precursor into a bNAb by stimulating favorable SHMs⁴¹. Example boosting immunogens reintroduce native Env glycans and heterogenous Env strains to develop bNAbs capable of overcoming steric glycan barriers and have heterologous-neutralizing activity⁴¹. The N276_{gp120} glycan on HIV-1 Env

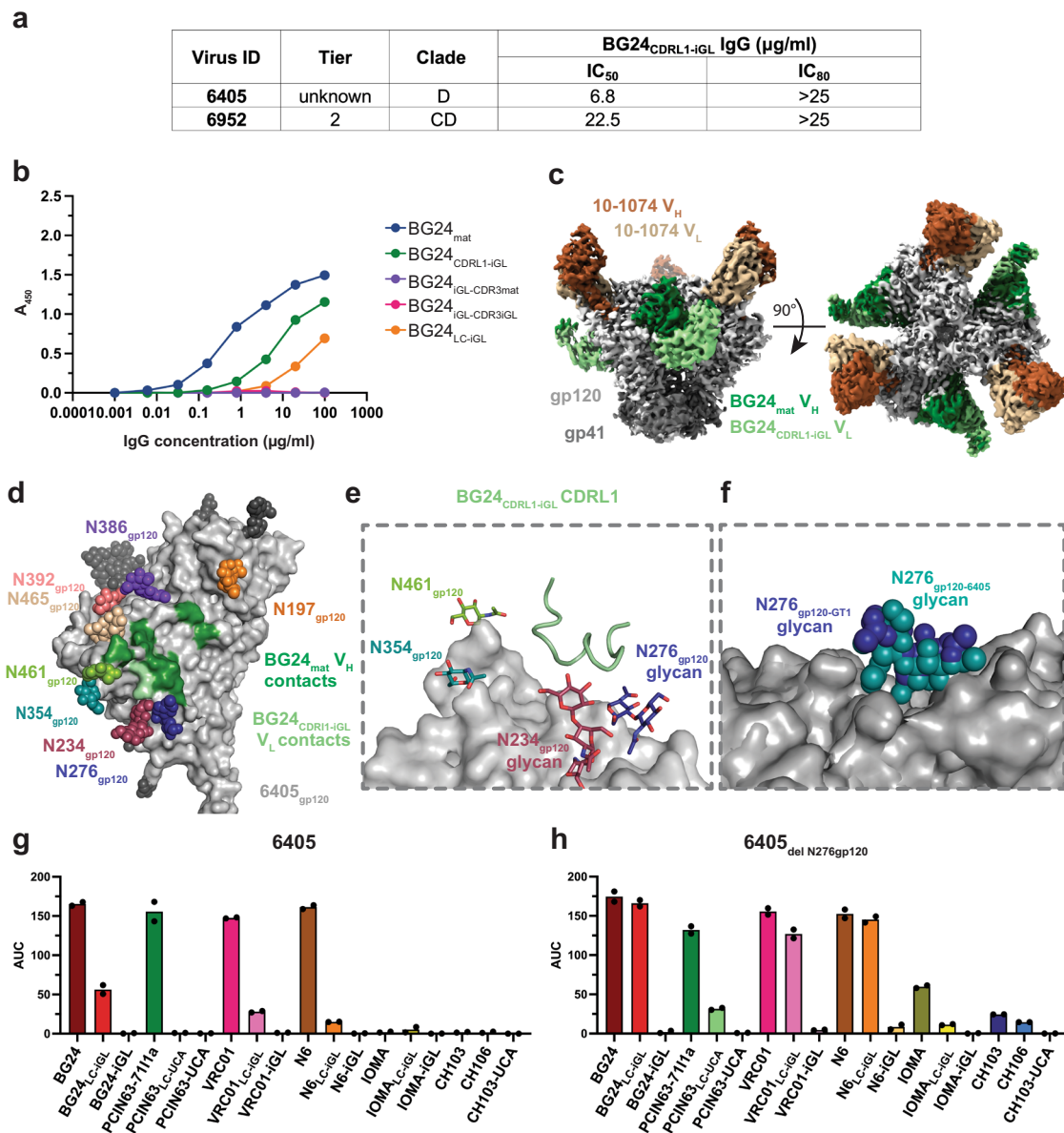


Fig. 5 | Non-engineered 6405 SOSIP recognizes BG24_{CDRL1-IgI}. **a** Summary of neutralization of 6405 and 6952 pseudoviruses by BG24_{CDRL1-IgI} IgGs. **b** ELISA to assess binding of BG24-derived Abs to 6405 SOSIP. Streptavidin plates were coated with randomly biotinylated SOSIPs and incubated with BG24-derived IgGs, at increasing concentrations. Values are shown as mean of two individual biological replicates ($n = 2$) with associated. **c** Side and top-down views of cryo-EM density of BG24_{CDRL1-IgI}-6405-10-1074. Highlighted in colors include: gp120 subunits (light gray), gp41 (dark gray), BG24_{CDRL1-IgI} V_H (dark green), and V_L (light green) domains, and 10-1074 V_H (dark brown) and V_L (light brown) domains. **d** Surface

contacts made by BG24_{CDRL1-IgI} V_H (dark green) and V_L (light green) on 6405 gp120 (light gray). **e** Cartoon representation for the CDRL1 of BG24_{CDRL1-IgI} (light green). **f** Alignment of GT1_{N276gp120} (light gray) and 6405 gp120s (light gray) in surface representation and N276 glycans (dark blue and deep teal) in sphere representation. **g**, **h** Summary for area under the curve (AUC) values derived from ELISAs that accessed binding of CD4bs IgGs to **g** 6405 and **h** 6405_{delN276gp120} SOSIPs. Streptavidin plates were coated with randomly biotinylated SOSIPs and incubated with CD4bs IgGs at increasing concentrations. Values are shown as mean of two individual biological replicates ($n = 2$). Source data are provided as a Source Data file.

provides a particularly difficult roadblock, as VRC01-like germline CDRL1s must become shorter or more flexible through SHM to avoid steric clashes^{23,27,28,32,48}. Several iterations of this approach have been tested in animal models; however, the elicitation of heterologous-neutralizing activity has not yet been accomplished^{40,49}.

BG24_{mat} represents a VRC01-class bNAb that can be targeted for germline-targeting approaches³⁰. BG24_{mat} has a fraction of the SHMs found in VRC01 and other VRC01-class bNAbs and maintains notable breadth and potency. Together with previous studies of the VRC01-class PCIN63 lineage and construction of a minimally mutated VRC01, our studies of BG24 suggest that high levels of SHM are not absolutely required for the development of VRC01-class Abs. Our cryo-EM

structures of the iGL precursors of BG24 bound to the priming immunogen GT1 contribute to understanding how VRC01-class bNAb precursors interact with immunogens. We found that VHI-2*02 germline-encoded residues make the predicted signature contacts with gp120, and the long germline CDRL1 is accommodated in the absence of the N276_{gp120} glycan in GT1, rationalizing removal of this glycan in a priming immunogen since modeling suggested the germline CDRL1 conformation would clash with the N276_{gp120} glycan. These observations validate the design of priming immunogens that nurture interactions with germline residues and remove the N276_{gp120} glycan from the CD4bs epitope. We further investigated how the glycan landscape of an immunogen affects germline binding, finding that

BG24_{iGL-LC} can evade clashes with the N276_{gp120} glycan when the BG24 HC includes bNAb features and the CD4bs epitope is only minimally glycosylated. Based on these observations, we propose that boosting immunogens might first aim to target mature HC features, and then introduce the N276_{gp120} glycan in a limited CD4bs glycan landscape before moving to a fully glycosylated Env landscape.

We also characterized the binding of BG24_{CDRL1-iGL} to the clade D 6405 Env, which suggested that some non-engineered HIV-1 Envs can accommodate some germline VRC01-class CDRL1s. In the case of BG24_{CDRL1-iGL}, accommodation of the N276_{gp120} glycan occurred through a helix-like conformation in the iGL CDRL1. Furthermore, ELISA data suggested that other VHI-2*02-derived bNAbs with iGL LCs can also bind to the 6405 Env. Taken together, we propose further investigation of the 6405 Env to identify properties of the CD4bs that led to the structural and biochemical observations reported here, which could be applicable to the design of CD4bs-targeting immunogens.

Methods

BG24_{iGL} constructs design

Genes encoding the IGHV1-2*02 and IGLV2-II*02 germline sequences with mature CDR3 loops were used to generate the BG24_{iGL-CDR3mat} Fab construct. For the BG24_{iGL-CDR3iGL} construct, amino acids in D- and J-gene regions were reverted based on inferred sequences using IMGT/V-QUEST. Mutations to BG24 IgG and Fab sequences were generated using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent).

Protein expression and purification

Fabs and IgGs were expressed and purified as previously described⁵⁰. Briefly, Fabs were expressed by transient transfection using the Expi293 expression system (ThermoFisher). Fab expression vectors contained genes of LC and the C-terminally 6x-His-tagged HC. The Fab and IgG proteins were purified from cell supernatants by Ni²⁺-NTA (GE Healthcare) and protein A affinity chromatography (GE Healthcare), respectively, followed by size exclusion chromatography (SEC) using a Superdex 200 10/300 column (GE Healthcare).

SOSIP.664 Env constructs contained the disulfide mutations 501 C and 605 C (SOS), I559P (IP), and the furin cleavage site mutated to six arginine residues (6R)³³. Genes encoding BG505 SOSIP.664v4.1-GT1 and 6405 SOSIPs were expressed using transient transfection of Expi293 cells (ThermoFisher) and purified as described previously⁵¹. The 6405 SOSIP construct contained gp120 residues 46–477 from the 6405 sequence, with the remaining gp120 residues derived from BG505 and the extracellular portion of the BG505 gp41⁵². Trimeric Env was separated from cell supernatants by PGT145 immunoaffinity chromatography and SEC using a Superose 6 10/300 column (GE Healthcare), as described⁵³.

X-ray crystallography

Purified BG24_{iGL-CDR3mat} Fab was concentrated to 8–15 mg/mL. Matrix crystallization screens were performed at room temperature using the sitting drop vapor diffusion method by mixing equal volumes of protein sample and reservoir using a TTP LabTech Mosquito robot and commercially-available screens (Hampton Research and Qiagen). Initial hits were optimized and crystals were obtained in 20% PEG 3350 at 20 °C. Crystals were cryo-protected in glycerol stepwise until 20% before being cryopreserved in liquid nitrogen.

X-ray diffraction data were collected to 1.4 Å for BG24_{iGL-CDR3mat} Fab at the Stanford Synchrotron Radiation Lightsources (SSRL) beamline 12-2 on a Pilatus 6 M pixel detector (Dectris). Data from a single crystal were indexed and integrated into XDS⁵⁴ and merged with AIMLESS in the CCP4 software suite⁵⁵. Structures were determined by molecular replacement in PHASER⁵⁶ using coordinates of the BG24_{mat} Fab (PDB 7UCE), after the removal of CDR loops and independent searches of the V_HV_L and C_HC_L domains. Models were refined using rigid body and

B-factor refinement in Phenix⁵⁷, followed by several cycles of iterative manual building in Coot⁵⁸ and real-space refinement with TLS groups in Phenix^{57,59} (Supplementary Table 2).

Assembly of protein complexes and cryo-EM sample preparation

Protein complexes for cryo-EM were generated by incubating a purified BG24_{iGL} Fab and the 10-1074 Fab with an Env trimer in a 3:1 Fab:trimer molar ratio and incubating at 4 °C overnight. The complex was then SEC purified over a Superdex 200 1/150 column (GE Healthcare). The peak corresponding to the complex was pooled and concentrated to 1.0 mg/mL. Quantifoil R2/2 400 mesh cryo-EM grids (Ted Pella) were prepared by glow-discharging for 1 min at 20 mA using a PELCO easiGLOW (Ted Pella). Fab-Env complexes (3 µL) were then applied to grids and blotted with Whatman No. 1 filter paper for 3–4 s at 100% humidity at room temperature. The grids were vitrified by plunge-freezing in liquid ethane using a Mark IV Vitrobot (ThermoFisher).

Cryo-EM data collection and processing

Data for single-particle cryo-EM were collected on either a Talos Arctica (BG24_{iGL-CDR3mat}-GT1-10-1074, BG24_{iGL-CDR3iGL}-GT1_{N276gp120}-10-1074, BG24_{CDRL1-iGL}-6405-10-1074) or a Titan Krios (BG24_{iGL-CDR3iGL}-GT1-10-1074) transmission electron microscope, operating at 200 and 300 kV, respectively. Movies were collected with beam-image shift over a single exposure per hole in a 3-by-3 pattern of 2 µm holes. For datasets collect on the Talos Arctica, movies were recorded in super-resolution mode on a Falcon III camera (Thermo Fisher) at 1.436 Å·pixel⁻¹ or a K3 camera (Gatan) at 0.4345 Å·pixel⁻¹. Movies obtained from samples on the Titan Krios were collected in super-resolution mode on a K3 camera (Gatan) equipped with a Bio-Quantum energy filter (Gatan) with a 20 eV slit width at 0.4327 Å·pixel⁻¹. The defocus range was set from 1.0 to 3.0 µm for each dataset.

The data processing workflow described below was performed similarly for all datasets using RELION^{60,61}. Movies were motion-corrected using MotionCor2⁶² after binning. GCTF⁶³ was used to estimate CTF, and micrograph power spectra that showed poor CTF fits or bad ice were removed. A subset of particles was manually picked and used for reference-free 2D classification. Classes representing the defined complex were used as references for RELION AutoPicking^{60,61} to generate 2D classes. Subsequent 2D classes were inspected, and 2D classes representing a defined complex were selected for 3D classification. An ab initio model was generated using cryoSPARC⁶⁴ using a subset of particles for each dataset and used as a reference in 3D classification, which assumed C1 symmetry. 3D classes representing a defined complex were selected for 3D auto-refinement and post-processing in Relion. Particles used in 3D refinement were then re-extracted and un-binned. Particles were then subjected to 3D classification with the map generated with un-binned particles used as a reference. Distinct classes representing a particular defined complex (C1 or C3 symmetric) were selected for 3D auto-refinement after masking out Fab C_HC_L domains. Iterative rounds of particle CTF refinement, particle polishing, 3D auto-refinement, and post-processing were used for each class to generate final maps. To improve the resolution of Fab_{LC} CDRL1s, a soft mask surrounding the Fab V_H-V_L-gp120 interface was created in chimera and used for local refinements in cryoSPARC to improve density in this region and allow for CDRL1 fitting and refinement. Resolutions were calculated in RELION using the gold-standard FSC 0.143 criterion⁶⁵. FSCs were generated by the 3DFSC program⁶⁶.

Cryo-EM model building and refinement

Model coordinates were generated by fitting reference gp120 (PDB 5T3Z), gp41 (PDB 5T3Z), 10-1074 (PDB 5T3Z), and BG24-derivative

Fabs (this study) chains into cryo-EM density with UCSF Chimera⁶⁷. Initial models were refined using the Phenix command *phenix.real_space_refine*^{57,59}. Sequence updates to the model and further manual refinement was conducted with Coot⁵⁸. Iterative rounds of Phenix auto-refinement and manual refinements were done to generate the final models (Supplementary Table 1).

Structural analyses

Structure figures were made using PyMol (Schrödinger LLC), UCSF Chimera⁶⁷, and UCSF ChimeraX^{68,69}. PyMol was used to calculate r.m.s.d. values after the pairwise alignment of C α atoms. PDBePISA⁷⁰ was used to calculate buried surface areas using a 1.4 Å probe. Calculations for gp120 BSA were for peptide components of gp120 and did not include glycan interactions. Defined interactions were assigned tentatively due to the low resolution of complexes using the following criteria: hydrogen bonds were assigned pairwise interactions that were less than 4.0 Å and with an A-D-H angle >90°, and van der Waals interactions were assigned as distances between atoms that were less than 4.0 Å.

TZM.bl neutralization assay

The neutralizing activities of BG24 CDRH2 mutant IgGs were measured using a luciferase-based TZM.bl assay according to standard protocols⁷¹. Each assay was performed in duplicate. Data were analyzed using Antibody Database (v2.0)⁷². Five-parameter curve fitting was used to determine 50% inhibitory concentrations (IC₅₀s), and non-specific activity was detected by testing against murine leukemia virus (MuLV).

Enzyme-linked immunosorbent assay

SOSIP trimers were randomly biotinylated following the manufacturer's guidelines using the EZ-Link NHS-PEG4-Biotin kit (Thermo Fisher Scientific). The Pierce Biotin kit (Thermo Fisher Scientific) was used to quantify biotin molecules per SOSIP protomer: biotin estimations ranged from 1 to 10 biotin molecules per SOSIP protomers. Streptavidin-coated 96-well plates (Thermo Fisher Scientific) were coated with 5 µg/mL of randomly biotinylated SOSIPs diluted in 3% BSA in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween20) and incubated at room temperature (RT) for 2 h. Plates were washed to remove unbound SOSIPs. Serial dilutions of IgGs were made in 3% BSA in TBS-T and applied to the plates. After a 2 h incubation at RT, plates were washed twice in TBS-T. Goat anti-human IgG Fc conjugated to horseradish peroxidase (Southern BioTech) was added at 1:8000 dilution for 30 min, followed by three washes with TBS-T. 1-Step™ Ultra TMB-ELISA Substrate Solution (ThermoFisher Scientific) was added for colorimetric detection, and color development was quenched with 1 N HCl. Absorbance was measured at 450 nm. Two independent, biological replicates ($n = 2$) were performed.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The atomic model generated for the X-ray crystallography structure of the BG24_{iGL-CDR3mat} Fab in this study has been deposited in the Protein Data Bank (PDB) under accession code [7UGM](#). The cryo-EM maps and atomic structures have been deposited in the PDB and/or Electron Microscopy Data Bank (EMDB) under accession codes [7UGN](#) and [EMD-26490](#) for BG24_{iGL-CDR3iGL-GT1-10-1074 Class I, [EMD-26491](#) for BG24_{iGL-CDR3iGL-GT1-10-1074 Class II, [7UGO](#) and [EMD-26492](#) for BG24_{iGL-CDR3mat-GT1-10-1074}, [7UGP](#) and [EMD-26493](#) for BG24_{iGL-LC-GT1-N276gp120-10-1074 Class I, [EMD-26494](#) for BG24_{iGL-LC-GT1-N276gp120-10-1074 Class II, [EMD-26495](#) for BG24_{iGL-LC-GT1-N276gp120-10-1074 Class III}, and [7UGQ](#) and [EMD-26496](#) for BG24_{CDRL1-iGL-6405-10-1074}. Local refinement}}}}

maps used to model CDRL1s of BG24-derivatives have been deposited with PDB and EMD accession codes for each respective structure. The ELISA data generated in this study are provided in the Supplementary Information and Source Data file. Source data are provided with this paper.

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- experiments. P.N.P.G. performed in vitro neutralization assays. K.A.D., C.O.B., and H.B.G. analyzed results. K.A.D. and P.J.B. wrote the manuscript with input from co-authors.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41467-022-33860-2>.

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Peer review information *Nature Communications* thanks Quentin Sattentau and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Acknowledgements

We thank J. Vielmetter, P. Hoffman, and the Protein Expression Center in the Beckman Institute at Caltech for expression assistance. Electron microscopy was performed in the Caltech Cryo-EM Center with assistance from S. Chen and A. Malyutin. We thank the Gordon and Betty Moore and Beckman Foundations for gifts to Caltech to support the Molecular Observatory (Dr. Jens Kaiser, Director) and the beamline staff at SSRL for data collection assistance. Use of the Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, is supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. DE-AC02-c76SF00515. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and by the National Institutes of Health, National Institute of General Medical Sciences (P41GM103393). The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of NIGMS or NIH. C.O.B. was supported by a Burroughs Wellcome PDEP fellowship and HHMI Hanna Gray Fellowship. This work was supported by the National Institute of Allergy and Infectious Diseases (NIAID) Grant HIVRAD P01 AI100148 (to P.J.B. and M.C.N.), the Bill and Melinda Gates Foundation Collaboration for AIDS Vaccine Discovery (CAVD) grant INV-002143 (P.J.B. and M.C.N.), and NIH P50 AI150464 (P.J.B.).

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K.A.D., C.O.B., H.B.G., T.S., M.C.N., and P.J.B. designed the research. K.A.D. performed protein purification, structural studies, and ELISA