Structural basis of broad ebolavirus neutralization by a human survivor antibody

Brandyn R. West1,2, Anna Z. Wec2,10,12, Crystal L. Moyer1,11, Marnie L. Fusco1, Philipp A. Ilinykh3,4, Kai Huang1,2, Ariel S. Wirchnianski2, Rebekah M. James5, Andrew S. Herbert5, Sean Hui1,12, Anna Z. Wec2,10,12, Crystal L. Moyer1,11, Marnie L. Fusco1, Philipp A. Ilinykh3,4, 1,9* 

The structural features that govern broad-spectrum activity of broadly neutralizing anti-ebolavirus antibodies (Abs) outside of the internal fusion loop epitope are currently unknown. Here we describe the structure of a broadly neutralizing human monoclonal Ab (mAb), ADI-15946, which was identified in a human survivor of the 2013–2016 outbreak. The crystal structure of ADI-15946 in complex with cleaved Ebola virus glycoprotein (EBOV GPcore) reveals that binding of the mAb structurally mimics the conserved interaction between the EBOV GP core and its glycan cap β17–β18 loop to inhibit infection. Both endosomal proteolysis of EBOV GP and binding of mAb FVM09 displace this loop, thereby increasing exposure of ADI-15946’s conserved epitope and enhancing neutralization. Our work also mapped the paratope of ADI-15946, thereby explaining reduced activity against Sudan virus, which enabled rational, structure-guided engineering to enhance binding and neutralization of Sudan virus while retaining the parental activity against EBOV and Bundibugyo virus.

EBOV and related members of the family Filoviridae cause outbreaks of highly lethal disease in humans. Ab therapeutics such as ZMapp, mAb142 and a three-mAb cocktail from Regeneron Pharmaceuticals (REGN-EB3) have recently been proposed for emergency use against Ebola virus disease7. However, the activity of these therapies is limited to EBOV and does not extend protection to the related virulent ebolaviruses Bundibugyo virus (BDBV) and Sudan virus (SUDV). Both BDBV and SUDV have caused sizeable outbreaks in the past and their potential for re-emergence remains unknown. No therapeutics are currently available for the treatment of BDBV and SUDV, which lends urgency to discovery and characterization of broadly active mAbs. The key obstacle for generation of such mAbs stems from limited amino acid sequence conservation among the glycoproteins of ebolaviruses, with only 50% amino acid identity shared between EBOV and SUDV, the two most prevalent ebolaviruses. Detailed characterization of known broadly neutralizing antibodies (bNAbs) and their modes of action will be critical to the design of next-generation broadly protective Ab cocktails and vaccines that elicit broadly protective responses.

All Ab therapeutics currently under development for Ebola virus disease target the ebolavirus surface glycoprotein, GP, which mediates viral entry into host cells by catalyzing viral membrane fusion in host cell endosomes2,5. During biogenesis, GP is post-translationally processed to yield GP1 and GP2 subunits (Fig. 1a), held together by a single disulfide bond, which associate into a trimer of GP1,2 heterodimers6. GP1 mediates host cell attachment and receptor recognition, whereas GP2 mediates fusion of the viral and host membranes2–12. The GP2 amino acid sequence, which includes the internal fusion loop (IFL), is highly conserved among ebolaviruses’ while the glycan cap and mucin-like domains of GP1 show higher sequence diversity. During infection, EBOV GP undergoes host-programmed disassembly mediated by endosomal cysteine proteases (cathepsins B and L), which shed the sterile bulk of the extensively glycosylated glycan cap and mucin-like domains and generate the cleaved GP intermediate (GPintermediate)13,14. This in turn unmask the receptor-binding site in GP1 and reveals other previously inaccessible regions of the GP core2,15,16. Engagement of the intracellular entry receptor Niemann–Pick C1 (NPC1) by the viral receptor-binding site is proposed to induce conformational rearrangements in GPintermediate that culminate in viral membrane fusion12,16–19. Antibodies targeting the functionally critical and conserved viral fusion machinery offer an efficacious mode of viral entry inhibition and have been shown to be strongly protective20–24.

The only pan-ebolavirus neutralizing Abs reported thus far target overlapping epitopes in the viral IFL21,23–26. In contrast, low-resolution negative stain reconstructions of the recently isolated human survivor mAb, ADI-15946, suggest that it may recognize a distinct footprint in the base region of GP, crosslinking the GP1 and GP2 subunits20. ADI-15946 potently neutralizes EBOV and BDBV but lacks neutralizing and protective activity against SUDV20. Accordingly, we sought to define the structural determinants of ADI-15946 activity in the context of its cognate antigen, EBOV GP, to characterize features of both its epitope and paratope that confer broad reactivity while investigating its limited activity against SUDV.
**Results**

**Crystal structure of EBOV GP\textsubscript{CL}–ADI-15946 complex.** We determined the crystal structure of ADI-15946’s fragment antigen binding (Fab) in complex with EBOV GP\textsubscript{CL} to 4.1 Å resolution (Fig. 1a,b and Table 1)\textsuperscript{20}. The structure was solved by molecular replacement using the previously published EBOV GP\textsubscript{CL} structure as a search model (PDB 5HJ3) and was refined to an \(R_{work} / R_{free}\) of 26.2/28.1% (Table 1)\textsuperscript{16}. The ADI-15946 Fab targets the base of a single GP1/GP2 protomer in the GP trimer at an approximately 45° angle relative to the surface of GP, with its constant domains directed downward toward the viral membrane (Fig. 1b).

The heavy and light chains each contribute roughly half of the overall ADI-15946 binding interface: ~55% from the heavy chain complementarity-determining region 3 (CDR H3), and the remaining 45% from the combined contributions of the light chain framework region 3 (FRL3) and CDRs 1, 2 and 3 (L1, L2 and L3, respectively) (Fig. 1b,c). Notably, CDRs 1 and 2 of the heavy chain (H1 and H2, respectively) do not participate in the Fab–GP\textsubscript{CL} interface (Fig. 1b,c). Instead, heavy chain recognition of GP\textsubscript{CL} is mediated exclusively by the CDR H3 that is 22 amino acids long, which bury roughly 465 Å\(^2\) of surface area on binding (Fig. 1b). The footprint of ADI-15946 partially overlaps those of the EBOV-monospecific binders KZ52, c2G4 and c4G7 (Supplementary Note 1)\textsuperscript{20}. However, ADI-15946 probably gains cross-reactivity from an upward shift of its footprint that allows it to target a highly conserved pocket above the upper boundary of the KZ52 epitope as suggested previously (Fig. 1d)\textsuperscript{20}.

**Modeling ADI-15946 interactions with GP2 and the glycan cap.** ADI-15946 heavy chain contacts include the strictly conserved lysine at position 510 in the GP2 N terminus—where a substitution to glutamic acid, K510E, was previously shown to give rise to escape from BDBV viral neutralization\textsuperscript{20}. We have confirmed that the same amino acid substitution in EBOV GP also leads to the loss of binding and neutralization phenotypes (data not shown). The electrostatic surface of ADI-15946 is complementary to that of GP, and our sidechain modeling suggests that the K510E substitution introduces a charged/steric clash with residues D100C and/or L100\(\beta\) of CDR H3 thereby explaining the loss of antiviral activity (Fig. 2a–c). We tested ADI-15946 variants containing alanine substitutions at either the D100\(\beta\) or the L100\(\beta\) positions for binding and neutralization and observed loss of both activities against recombinant vesicular stomatitis virus (rVSV) particles bearing BDBV GP K510E (Fig. 2d). Additionally, the ADI-15946 D100\(\beta\)A variant lost activity to wild-type rVSV-BDBV GP probably due to the loss of stabilizing interactions between the D100\(\beta\) sidechain and the backbone of CDR H3 (Fig. 2d). These results indicated that interaction with the conserved K510 residue is a key contributor to ADI-15946’s breadth and that overcoming the K510E escape mutation would be difficult without extensive mutagenesis of the antibody paratope.

Wec et al.\textsuperscript{20} have shown that ADI-15946 inhibits GP proteolysis in vitro, a key step in unmasking of the viral receptor-binding site that is indispensable for infection. The left boundary of ADI-15946’s footprint, formed by light chain FRL3, is in immediate proximity to the β13–β14 loop that passes over the IFL and is cleaved by endosomal cathepsins during viral entry (Supplementary Fig. 1)\textsuperscript{13,14}.
Overlay of our structure with uncleaved GP (PDB 5JQ3) shows that ADI-15946 binding probably impedes protease access to this loop, thus explaining ADI-15946 inhibition of GP proteolysis (Supplementary Fig. 1). We next tested the effect of FVM09 on ADI-15946 neutralization of rVSV-EBOV GPW291R compared to rVSV bearing wild-type EBOV GP (GPWT) (Fig. 3e). We first tested competition between ADI-15946 and FVM09 using biolayer interferometry-based competitive binding assays and found that both mAbs could bind to EBOV GP simultaneously (Supplementary Fig. 2). Given the sizeable contribution of CDR H3 to the binding interface, the slower dissociation rate against GPCL may reflect a mechanism by which specific combinations of glycan cap- and base-binding mAbs can synergize to interdict viral entry. The enhancement of ADI-15946 binding and neutralization with mAb FVM09 may relate to the transduction of the 310 pocket from the conserved pocket by introducing charged and steric clashes with the GP2 residue asparagine 512. Consistent with this hypothesis, we observed a 10-fold enhancement in ADI-15946 binding and neutralization of rVSV-EBOV GPW291R compared to rVSV bearing wild-type EBOV GP (GPWT) (Supplementary Table 1). We then tested ADI-15946 neutralization against rVSVs bearing an EBOV GAPβ17–18 variant in which the β17–β18 loop has been deleted (residues 187–198). Removal of the β17–β18 loop had no apparent adverse effects on viral infectivity or replication in vitro (data not shown). We observed a 10-fold increase in neutralization efficiency of rVSV-EBOV GPAPβ17–18 compared to GPWT (Fig. 3c). The intermediate neutralization potency of rVSV-EBOV GPW291R compared to GPWT and GPWT suggests that displacement of the β17–β18 loop from the 310 pocket is the limiting factor for binding of ADI-15946 to EBOV GP (Fig. 3e).

**Enhancement of binding and neutralization with mAb FVM09.** Previous work suggests that a non-neutralizing mAb, FVM09, recognizes and ‘peels away’ the β17–β18 loop from the base, thereby enhancing neutralization by the base-binding mAb c2G4 and c2G5. Given the more profound role of the β17–β18 loop in restricting access of ADI-15946 to its base epitope (Supplementary Table 2), we evaluated the hypothesis that FVM09 could also work together with ADI-15946 to neutralize virus with enhanced potency (Supplementary Fig. 2). We first tested competition between ADI-15946 and FVM09 using biolayer interferometry-based competitive binding assays and found that both mAbs could bind to EBOV GP simultaneously (Supplementary Fig. 4). We next tested the effect of FVM09 on ADI-15946 neutralization of rVSV-EBOV GP or authentic EBOV in the presence of three fixed concentrations of FVM09. FVM09 potentiated ADI-15946 neutralization in a concentration-dependent manner by >10-fold, but had no effect on neutralization by KZ52 (Fig. 4a). Conversely, titration of FVM09 into a constant, subneutralizing concentration of ADI-15946 also showed dose-dependent enhancement of ADI-15946 neutralizing activity (Fig. 4b). In contrast, KZ52 neutralization was reduced in the same assay, perhaps because KZ52 binds along the surface of the β17–β18 loop whereas ADI-15946 binds beneath the β17–β18 loop (Fig. 4b and Supplementary Fig. 5). We observed similar neutralization enhancement trends with authentic EBOV (Fig. 4c,d), and to a lesser extent, with rVSV-SUDV GP (Fig. 4e,f).

The functional importance of the strict conservation of the amino acid sequence in the 310 pocket and the β17–β18 loop among ebolavirus GPs remains unknown, but it may relate to the transduction of conformational changes in GP on NPC1 binding (Fig. 3a and Supplementary Note 2). Our results demonstrate that rare neutralizing antibodies such as ADI-15946 can access and exploit this cryptic site of broad ebolavirus vulnerability to achieve neutralization, presumably by interfering with the region’s functional role in viral entry. The enhancement of ADI-15946 binding and neutralization in the presence of β17–β18 loop binders such as FVM09 may reflect a mechanism by which specific combinations of glycan cap- and base-binding mAbs can synergize to interdict viral infection during a natural polyclonal immune response (Fig. 4g).

**Table 1 | Data collection and refinement statistics**

| EBOV GPcl-ADI-15946 Fab (PDB 6MAM) |
|-------------------------------------|
| **Data collection**                  |
| Space group                         | P4,22 |
| Cell dimensions                     | 182.27, 182.27, 262.01 |
| a, b, c (Å)                         | 90, 90, 90 |
| Resolution (Å)                      | 48.54–4.10 (4.25–4.10) |
| Rmerge                              | 0.20 (1.83) |
| Rfree                               | 0.05 (0.46) |
| I/σ(I)                              | 14.6 (1.8) |
| CC1/2                               | 1.00 (0.70) |
| Completeness (%)                    | 99.6 (100.0) |
| Redundancy                          | 131 (13.4) |
| **Refinement**                      |
| Resolution (Å)                      | 48.54–4.10 (4.25–4.10) |
| No. reflections                     | 35,153 (3,458) |
| Rwork / Rfree                       | 0.262 / 0.281 |
| No. atoms                           | 16,525 |
| Protein                             | 172 |
| Ligands (glycans)                   | 114 |
| B factors                           | 107 |
| R.m.s. deviations                   | 0.01 |
| Bond lengths (Å)                    | 0.61 |
| Bond angles (°)                     | 0.41 |

*Diffraction data were collected from a single crystal. Values in parentheses are for highest-resolution shell.*

and dissociation (koff) rates of ADI-15946 to EBOV GPWT and GPcl showed that the improved binding to GPcl is primarily driven by an over 1,000-fold slower koff and a modest improvement of the association rate (10-fold increased kass) (Fig. 3f, Supplementary Fig. 3 and Supplementary Table 2). Given the sizeable contribution of CDR H3 to the binding interface, the slower dissociation rate against GPcl probably results from its unobstructed access to the 310 pocket.

To specifically probe the importance of the β17–β18 loop’s hydrophobic packing into the 310 pocket to the shielding of the ADI-15946 epitope, we first tested ADI-15946 binding and neutralization against rVSVs bearing an EBOV GP variant with an arginine substitution at position 291 (rVSV-EBOV GPW291R). Modeling of the arginine sidechain suggested that the W291R substitution would displace the β17–β18 loop from the conserved pocket by introducing charged and steric clashes with the GP2 residue asparagine 512. Consistent with this hypothesis, we observed a 10-fold enhancement in ADI-15946 binding and neutralization of rVSV-EBOV GPW291R compared to rVSV bearing wild-type EBOV GP (GPWT) (Supplementary Table 1). We then tested ADI-15946 neutralization against rVSVs bearing an EBOV GPAPβ17–18 variant in which the β17–β18 loop was deleted (residues 187–198). Removal of the β17–β18 loop had no apparent adverse effects on viral infectivity or replication in vitro (data not shown). We observed a 10-fold increase in neutralization efficiency of rVSV-EBOV GPAPβ17–18 compared to GPWT (Fig. 3c). The intermediate neutralization potency of rVSV-EBOV GPW291R compared to GPcl and GPWT suggests that displacement of the β17–β18 loop from the 310 pocket is the limiting factor for binding of ADI-15946 to EBOV GP (Fig. 3e).
Molecular determinants for somatic maturation of ADI-15946. To delineate the molecular basis of ADI-15946’s broad activity, we assigned its light and heavy chain variable domain sequences to their most probable inferred germline progenitors (IGL) (Fig. 5a and Supplementary Note 3). We found that reversion of three residues introduced by somatic hypermutation (SHM) in the variable heavy chain domain to germline (light chain, LC; heavy chain, HC) (LCWT:HCIGL; CDR H3 is retained fully mature) had no appreciable impact on binding of full-length or cleaved EBOV, BDBV or SUDV GP or neutralization of rVSVs bearing EBOV, BDBV or SUDV GP (Fig. 5b,c and Supplementary Fig. 6). These findings agree with our structural observations, since only CDR H3 participates in binding to EBOV GPCL (Fig. 1b).

Reversion of 10 SHM-introduced residues in the variable heavy chain domain outside of CDR H3 (LC IGL:HCWT; residues 3, 4, 27, 28, 31, 53, 67, 72, 87, 104) had a profound impact on neutralization of rVSVs bearing EBOV, BDBV or SUDV GP (Fig. 5b,c and Supplementary Fig. 6). Both the LC IGL:HCWT and the combined LC IGL:HCWT Ab variants were non-neutralizing against rVSV-EBOV GPWT, despite retaining a fully mature CDR H3 and CDR L3, suggesting that LC contacts outside of CDR H3 make key contributions to GP recognition (Fig. 5c). The LC IGL:HCWT and the LC IGL:HCWT germline-approximating Ab variants retained some neutralizing activity against EBOV GPCL and BDBV GPCL, probably due to improved access of CDR H3 to the 310 pocket (Fig. 5c and Supplementary Table 1). However, LC IGL:HCWT neutralized the rVSV-EBOV GPWT variant more potently than rVSV-EBOV GPWT, which suggests that additional LC contacts that are absent in GPWT, probably between the Ab and the glycan cap, contribute to ADI-15946’s activity against GPWT (Supplementary Figs. 1 and 6 and Supplementary Table 1).

Enhancement of ADI-15946 activity against SUDV. Our analysis of the conservation in ADI-15946’s epitope shows that limited SUDV reactivity and lack of activity against Reston virus (RESTV) probably arise from the amino acid sequence divergence on the edges of the ADI-15946’s epitope (Fig. 2a, b). The K510E escape mutation probably clashes with ADI-15946 CDR H3. a, Stereoview of the EBOV GPCL–ADI-15946 complex (left; ADI-15946 in orange, and GPCL in dark and light teal for GP1 and GP2, respectively) and electrostatic surface potential (right; color scale shown in c) showing that residue K510 of GP2 binds into a negatively charged pocket created by ADI-15946 CDR H3. b, Similar views to a, with modeling of an escape mutant of ADI-15946, GP K510E, suggesting that K510E clashes with CDR H3 and introduces conflicting negative charge into the CDR H3 pocket. c, Open-book representation of EBOV GPCL and ADI-15946 showing electrostatic surface potential colored according to included scale. GPCL is shown on the left with the epitope outlined in black. ADI-15946 is shown on the right with the paratope outlined in black. kb, Boltzmann’s constant (1.3806504 × 10–23 J K–1). T, temperature (310 ºK). e, charge of an electron (1.60217646 × 10–19 C). d, Binding and neutralization assays showing the capacity of ADI-15946 variants containing either the D100A or the L100A mutation to bind to rVSV-BDBV GP (wild type, WT or K510E) in an ELISA (top, mean ± s.d., n = 4 biologically independent samples) and neutralize infection by these viruses (bottom, mean ± s.d., n = 6 biologically independent samples). Electrostatic surface potentials in a and c were generated using the APBS plugin with Pymol. A450 nm, absorbance at 450 nm.
its footprint (Fig. 6f and Supplementary Note 2). There are only five nonconserved residues across all five ebolavirus GPs involved in the interface with ADI-15946, all located in GP2: N506, N514, H516, L547 and H549. These are predominantly contacted by CDR L2 and FRL3 (Fig. 6f). However, CDR H3 contacts one key residue in EBOV GP (Asn 506) that is not conserved in SUDV GP (Arg 506). Using this information, we separately mutated nearby residues in ADI-15946 CDR H3, R100 and Y100A to alanine to reduce steric and charge clashes with SUDV R506. The ADI-15946 mutant Y100AA showed decreased binding to both SUDV and BDBV GP compared to wild-type ADI-15946 (Supplementary Fig. 7). However, the mAb variant containing R100A, called 46M1, showed...
increased binding to SUDV GP ectodomain by ELISA and slightly improved neutralization of authentic virus compared to the parental antibody (Fig. 6b–e and Supplementary Fig. 7).

Structural alignment of additional Fab-GP complexes enabled comparison of the binding features of the ADI-15946 paratope with those of EBOV-monospecific mAbs KZ52, c2G4 and c4G7, which have overlapping epitopes with that of ADI-15946 (Supplementary Fig. 5 and Supplementary Note 1)\(^{\#}\). We observed that the heavy chain paratope of c4G7 places a tyrosine residue in a similar position and orientation to that of the ADI-15946 LC residue F67 and another tyrosine residue proximal to ADI-15946 LC residue S65. We then attempted to mimic this double tyrosine motif by incorporation of light chain S65Y and F67Y mutations into ADI-15946. The construct bearing heavy chain R100A and light chain S65Y is called variant 46M2, and ADI-15946 bearing the R100A/S65Y pair plus LC substitution F67Y is called variant 46M3 (Fig. 6a). The 46M2 and 46M3 antibodies showed enhanced binding of SUDV, while maintaining parental binding to EBOV and BDBV GPs (Fig. 6b).

The 46M2 and 46M3 antibodies also exhibited a 16- to 33-fold improvement in their capacity to neutralize SUDV GP-bearing rVSV and authentic SUDV respectively, while retaining neutralization of EBOV and BDBV (Fig. 6c–d and Supplementary Fig. 7). Slight variations were observed in the neutralization profiles of the affinity variants versus the parent ADI-15946: we observed a roughly two-fold increase in neutralization for the affinity variants against wild-type ADI-15946 and a two-fold decrease in neutralization for the affinity variants versus the parent ADI-15946: we observed a roughly two-fold increase in neutralization for the affinity variants against wild-type ADI-15946 and a two-fold decrease in neutralization for the affinity variants against EBOV compared to wild-type ADI-15946 and a two-fold decrease in neutralization for the affinity variants against EBOV compared to wild-type ADI-15946 (Fig. 6d,e and Supplementary Tables 1 and 3). Further, the variants and the parental antibody similarly recognize the conserved 310 pocket, as all are mutations introduced in the 46M2 and 46M3 variants resulted in improved binding and neutralization of SUDV and confirmed the importance of the LC FR3 region to the binding interface.

Fig. 4 | mAb FVM09 potentiates ADI-15946 neutralization of EBOV GP in a dose-dependent manner. a, Infection assays showing that addition of increasing concentrations of ADI-15946 (left), but not KZ52 (right), to fixed concentrations of FVM09 (5, 20 or 80 nM) promotes rVSV-EBOV GP neutralization. The reciprocal fold change in neutralization half-maximal inhibitory concentration (IC\(_{50}\)) is shown (inset). b, Infection assays showing that addition of increasing concentrations of FVM09 to a fixed, subneutralizing concentration of ADI-15946 enhances rVSV-EBOV GP neutralization. The same experiment against KZ52 shows inhibition of neutralization as the concentration of FVM09 is increased. c-f, Infection assays as in a, b, with authentic EBOV showing similar trends; this trend is present but less pronounced with rVSV-SUDV GP (c,d). Data in a-f are mean ± s.d., n = 6 biologically independent samples. g, A cartoon showing the proposed relationship between FVM09 binding and subsequent exposure of the ADI-15946 binding site.
extracellular and intracellular viral species. In contrast, other potent neutralizers that target the GP base, such as KZ52 and the ZMapp components c2G4 and c4G7, have been shown to lose their activity against GPCL and therefore have activity only against the extracellular virus\cite{3,27}. The unique mechanism of neutralization by ADI-15946 may involve binding of ADI-15946 in the $3_{10}$ pocket, which in turn prevents rearrangement of GP1 residues 71–75 on binding of GPCL to NPC1 by mimicking the hydrophobic interactions of the $B1\overline{7}$–$B1\overline{8}$ loop that packs into the pocket in GPCL (Fig. 3a,b)\cite{12}. In addition, ADI-15946 may also inhibit conformational changes required for membrane fusion by anchoring to the GP1-GP2 interface across the IFL thereby preventing its unraveling from the GP1 core during fusion triggering.

We also uncovered potentially synergistic neutralization between ADI-15946 and FVM09, a non-neutralizing glycan cap-binding antibody. FVM09 was previously shown to enhance binding activity of another glycan cap mAb, m8C4, yet reduce binding of the base-targeting mAb KZ52—the latter being consistent with our own observations\cite{30}. As with ADI-15946, mutations of the $B1\overline{7}$–$B1\overline{8}$ loop also enhanced m8C4's binding and neutralizing activity in vitro\cite{30}. The exact mechanism of FVM09 and m8C4 cooperation is difficult to discern without structural details of the binding interface between the two mAbs and GP. In the case of cooperativity between FVM09 and ADI-15946, however, we propose that FVM09 primarily plays a supporting role by exposing the $3_{10}$ pocket thereby enhancing ADI-15946 binding and neutralization. It is possible that co-administration of FVM09 and ADI-15946 would lead to enhancement in protective efficacy in vivo. However, given that FVM09 is a non-neutralizing and non-protective antibody, viral escape from ADI-15946 neutralization would render the two antibody combination non-protective. Follow up studies will be required to fully evaluate the therapeutic usefulness of such antibody combinations.

Our analysis of the contributions of somatic hypermutation to the binding and neutralization properties of ADI-15946 indicated that only a limited number of amino acid substitutions in the germline antibody sequence were required for binding of this conserved GP site and that many of these mutations occurred in the framework region. Klein et al. show that somatic mutations in the immunoglobulin framework region of antibodies against HIV-1 indicated that only a limited number of amino acid substitutions in the germline antibody sequence were required for binding of this conserved GP site and that many of these mutations occurred in the framework region. Klein et al. show that somatic mutations in the immunoglobulin framework region of antibodies against HIV-1 enhanced affinity by decreasing the dissociation rate and are generally necessary to achieve broad neutralization\cite{31}. We found that, similar to bNAbS against HIV-1, ADI-15946 also required somatic maturation of the framework region, which bestowed the mAb with an improved dissociation rate and broad neutralizing activity of ebolaviruses\cite{31}. Specifically, as a result of SHM, a serine at position 67 of ADI-15946 FRL3 was replaced with an aromatic phenylalanine sidechain that interacts with GP2 residue H516 (100% conserved between EBOV, BDBV and SUDV). Interestingly, the non-pathogenic RESTV encodes a tyrosine at position S16 in GP2, which may play a role in the inability of ADI-15946 to neutralize RESTV due to steric hindrance with the antibody phenylalanine 67 (Supplementary Fig. 7e and Supplementary Note 2).

This structure provided the molecular details of the paratope required for rational engineering of the parent mAb to expand its activity against SUDV. We showed that structure-guided substitutions, two in the FRL3 and one in CDR H3, enhanced both binding and neutralization activity against SUDV without loss of affinity against EBOV or BDBV. However, follow-up work will be required to evaluate additional mAb variants and their biophysical properties to ensure favorable in vivo pharmacokinetics and protective efficacy against SUDV since, unlike natural antibodies, mAbs generated through in vitro engineering have not undergone immune tolerance selection. Our work also details structural features of GP recognition by a broadly active anti-ebolavirus antibody and suggests specific strategies such as the use of GPCL (lacking the glycan cap) or GP variants with $B1\overline{7}$–$B1\overline{8}$ loop deletion to enable targeting
Fig. 6 | Structure-guided affinity maturation of ADI-15946. a, Molecular models showing the locations of mutations in ADI-15946 variants 46M1, 46M2 and 46M3 in relation to the surface of GP. CDRs are illustrated in dark orange for the heavy chain and light orange for the light chain, respectively; engineered side chains that differ from wild type are colored in green. GP1 and GP2 are shown as a gray and a white surface, respectively. b, Binding assays of recombinant EBOV, BDBV and SUDV GP ectodomains by the indicated ADI-15946 variants determined by ELISA. Data are mean ± s.d., n = 3 biologically independent samples. c, Neutralization assays of authentic EBOV, BDBV and SUDV by the indicated ADI-15946 variants. Data are mean ± s.d., n = 6 biologically independent samples. d, e, Heat maps for neutralization potency (IC\textsubscript{50}) of each ADI-15946 variant against rVSVs (d) and authentic filoviruses (e). In d, neutralization of rVSVs bearing full-length GPs and cleaved GPs is shown on the top and bottom, respectively. f, Molecular surface of EBOV GP\textsubscript{cl} with the ADI-15946 footprint outlined in orange. Differences at five sites are listed on the left. The panel on the right shows which CDRs are in proximity to these nonconserved sites. TAFV, Tai Forest virus. g, Neutralization assays of rVSV-EBOV GP by 46M3 in the presence of increasing concentrations of FVM09 (0–100 nM). Increasing amounts of FVM09 improved 46M3 neutralization. Data are mean ± s.d., n = 6 biologically independent samples.
of the highly conserved site in the design of vaccines and immunotherapeutics. Like ADI-15946, the pan-ebolavirus mAb ADI-15878 also binds into a cryptic pocket at a distinct site in the GP base, that is typically occupied by the N-terminal tail of GP2 when not bound by the mAb. Future ebolavirus vaccine designs may strive to enhance the exposure of both of these vulnerable pockets on a single antigen by developing an immunogen that combines β17–β18 loop deletion with a truncated GP2 N terminus. Such immunogens may help elicit bNAbbs such as ADI-15946 at a higher frequency while minimizing potential for viral escape by eliciting bNAbbs towards multiple broadly conserved sites, both located proximal to the functionally critical region of the GP fusion loop. A broadly reactive vaccine would afford increased preparedness against ebolavirus disease regardless of the specific virus responsible for an outbreak.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41594-019-01914-4.

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Author contributions
B.R.W., A.Z.W., C.L.M., M.L.F., P.A.I., K.H., A.S.W., R.M.J., A.S.H., S.H., E.G., K.A.H. and S.K. carried out the research. B.R.W., A.Z.W., K.C. and E.O.S. designed the study. M.J.A. contributed materials. L.M.W., J.M.D., A.B., K.C. and E.O.S. supervised the research. B.R.W., A.Z.W., K.C. and E.O.S. drafted the manuscript. B.R.W., A.Z.W., K.C. and E.O.S. edited the manuscript. All authors analyzed data and commented on the drafts.

Competing interests
M.J.A. has stock in Integrated Biotherapeutics, a company developing antibody therapeutics for ebolavirus disease. A.Z.W., E.G. and L.M.W. are employees and equity holders of Adimab. K.C. and E.O.S. are members of the Scientific Advisory Board of Adimab. K.C. and J.M.D. are members of the Scientific Advisory Board of Integrated Biotherapeutics. M.J.A. has stock in Integrated Biotherapeutics, a company developing antibody therapeutics for ebolavirus disease. A.Z.W., E.G. and L.M.W. are employees and equity holders of Adimab. K.C. and E.O.S. are members of the Scientific Advisory Board of Adimab. K.C. and J.M.D. are members of the Scientific Advisory Board of Integrated Biotherapeutics.

Additional information
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Methods
Cloning. Mutants were introduced into parental ADI-15946 by site-directed mutagenesis using QuickChange (Agilent), with all mutations confirmed by sequencing (Éton).

Protein expression and purification. Expression and purification of EBOV GP CL was carried out as described previously40. Briefly, ebolavirus GP (lacking the mucin domain residues 312–462) was produced by stable expression in Drosophila melanogaster S2 cells. Effectene (Qiagen) was used to transfect S2 cells with a modified pMT-puro vector plasmid containing the GP gene of interest, followed by stable selection of transfected cells with 6 μg/ml puroycin. Cells were cultured at 27°C in complete Schneider’s medium for selection and then adapted to Insect Xpress medium (Lonza) for large-scale expression in 2-L Erlenmeyer flasks. Secreted GP ectodomain expression was induced with 0.5 mM CuSO4, and to Insect Xpress medium (Lonza) for large-scale expression in 2-L Erlenmeyer flasks. Secreted GP ectodomain expression was induced with 0.5 mM CuSO4, and purified according to the protocol for GPCL with the exception that SEC was performed with Superdex 200 (GE) size exclusion chromatography (SEC) in 10 mM Tris-buffered saline (Tris-HCl, pH 7.5, 150 mM NaCl (TBS)). EBOV GP CL was produced by incubation of 1 mg GP with 0.02 mg thrombin overnight at room temperature in TBS containing 1 mM CaCl2, and purified using Superdex 200 SEC.

ADI-15946 Fab used for crystallization experiments was cloned into a modified pMT-puro vector with a heavy chain C-terminal Strep-tag, and then expressed and purified according to the protocol for GP CL with the exception that SEC was performed with a Superdex 75 column (GE). ADI-15946 IgG for ELISA and neutralization assays were produced in ExpHCHO cells (ThermoFisher Scientific) and purified via Protein A chromatography according to the standard ThermoFisher ExpChIP protocol for a 25 ml culture volume. Cells were pelleted 8 days post transfection by centrifugation and concentrated to 4.2 mg ml⁻¹ in TBS. The crystal drops consisted of a 1:1 ratio of protein/well solution. Crystals grew over the course of 1 month in 0.2 M sodium citrate tribasic dihydrate pH 8.2 and 20% polyethylene glycol 3350. Crystals were cryoprotected with 20% glycerol and flash frozen in liquid nitrogen for storage and shipping. Diffraction data was collected remotely on SSLR beamline 12-2 on a pilatus 6 M detector23–25. Data was processed using XDS26, and the structure was determined using molecular replacement with PHASER27 in the CCP4 suite28, using the structure of EBOV GP CL (PDB 5HJ3) as an initial search model29. Iterative rounds of model building were performed using Coot30, and each round was refined with Phenix31. Five percent of the data was set aside before refinement for the Ramachandran calculations for each data set32. The statistics and stereochemistry of the crystal structure were checked using the MolProbity server33. Structural figures were rendered using Open Source PyMOL (PyMOL Molecular Graphics System, v1.7.0.0; Schrödinger, LLC).

Structural alignment and visualization of ebolavirus glycoproteins. Alignment was performed using clustalomega on uniprot with the following protein sequences: Zaire ebolavirus: Q05320, Bundibugyo ebolavirus: B8CKN0, Sudan ebolavirus: Q66814, Tai Forest ebolavirus: Q66810, Reston ebolavirus: Q66799. Sequence conservation was calculated according to EBOV GP and visualized using the Espript server (http://espript.ibcp.fr) and colored according to the percent equivalent scoring function with a cutoff of 70% (ref. 34).

Determination of inferred germline progenitors. ADI-15946 heavy and light chain sequences were aligned to their nearest predicted germline progenitors using IGH. Amino acid substitutions in the variable region of the heavy and light chain outside of CDR H3 and CDR L3 of the mature antibody were reverted to germline-encoded amino acids. Germline-reverted sequences of variable heavy and light chain domains were then ordered as gBlocks with overhangs to allow for homologous recombination into Saccharomyces cerevisiae.

Anti-GP mAb ELISAs. High-binding 96-well ELISA plates (Corning) were coated with 50 μl GP antigens in phosphate-buffered saline (PBS) at 4 μg/ml and allowed to bind for 1 h at room temperature. After washing, the wells were blocked with PBS containing 3% bovine serum albumin (PBSA) for 1 h at room temperature, followed by washing then incubation with ADI-15946 or one of its mutant derivatives in serial dilutions of PBS. A horseradish-peroxidase conjugated anti-human secondary antibody (Santa Cruz Biotechnology) was added and allowed to bind for 1 h at room temperature and then subsequently detected by ultra-TMB (3,3',5,5'-tetramethylbenzidine) substrate (ThermoFisher Scientific). Optical density was measured at 450 nm, and absorbance readings were subjected to a nonlinear regression analysis (GraphPad Prism software) to generate binding curves and calculate a half-maximum effective concentration value. ELISA assays were performed in triplicate, across seven five-fold dilutions, beginning at 1 ng ml⁻¹.

Biolayer interferometry assays. The Octet Red system (FortéBio, Pall) was used to determine the binding properties of different IgGs to various forms of EBOV GP. Anti-human Fc (AHF) capture sensors (FortéBio) were used for initial mAb loading at 25 mg ml⁻¹ in 1x kinetics buffer (PBS supplemented with 0.002% Tween-20 and 1 mg ml⁻¹ of BSA). Binding to GP was carried out across two-fold serial dilutions of EBOV GP CL or GP CL₂. The baseline and dissociation steps were carried out in the 1x kinetics buffer as per the instrument manufacturer’s recommendations. Kinetic binding data in all cases are adequately and accurately described by a 1:1 binding model, but given the bivalent nature of the IgG (immobilized) and the trimeric state of GP (analyte), the association stoichiometry is likely to be more complex. Thus, we refer to this ratio as apparent Kᵣ (Kᵥ th) throughout.

rSVS neutralization assays. Neutralization of rSVS: recombinant VSV expressing both enhanced green fluorescent protein (EGFP) and recombinant surface GP (rSVS-EBOV GP) in place of VSV G have been previously described41.6. Vero cells were seeded at 6.0 × 10⁴ cells per well and cultured overnight in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 100 U per ml of penicillin and 100 μg ml⁻¹ streptomycin at 37°C and 5% CO₂. The next day, virus was incubated with serial three-fold antibody dilutions, beginning at 350 μl (50 μg ml⁻¹) in serum free DMEM for 1 h at room temperature before infecting Vero cell monolayers in 96-well plates. The virus was incubated with the cells in 50 μl DMEM supplemented with 2% FBS, 100 μl per ml of penicillin and 100 μg ml⁻¹ streptomycin at 37°C and 5% CO₂, for 14–16 h before the cells were fixed and the nuclei stained with Hoechst 33342. rSVS infectivity was measured by counting EGFP-positive cells in comparison to the number of cells indicated by nuclear staining using a CellInsight CX5 automated microscope and accompanying software (ThermoFisher Scientific).

Authentic virus neutralization assays. Neutralization at BSL-4 was tested against replication-competent infectious IgG expressing EBOV and chimeric EBOV/BDBV-GP and EBOV/SUDV-GP constructs (referred to as EBOV, BDBV and SUDV, respectively) in HTS format, as previously described48. The neutralization assays were performed using Vero-E6 cells obtained from ATCC and maintained in minimal essential medium (MEM) (ThermoFisher Scientific) supplemented by 10% FBS (HyClone) and 1% penicillin-streptomycin at 5% CO₂, 37°C. Neutralization assays were performed in triplicate, across 12 four-fold dilutions, starting from 200 μg ml⁻¹.

Authentic virus cooperativity assays. The authentic Ebola virus/h. sapiens/ tc/ COD/1995/Kikwit-9510621 (EBOV/Kik-9510621; EBOV-Zaire 1995)49, was used in this study. Antibodies were diluted to indicated concentrations in culture media and incubated with EBOV for 1 h. Vero cells were exposed to antibody/virus inoculum at an MOI of 0.2 plaque-forming unit per cell for 1 h. Antibody/virus inoculum was then removed and fresh culture media was added. At 48 h post-infection, cells were fixed with formalin, and blocked with 1% bovine serum albumin. EBOV-infected cells and uninfected controls were incubated with EBOV GP specific mAb KZ5250. Cells were washed with PBS before incubation with goat anti-human IgG conjugated to Alexa 488. Cells were counterstained with Hoechst 33342 stain (Invitrogen), washed with PBS and stored at 4°C. Infected cells were quantitated by fluorescence microscopy and automated image analysis. Images were acquired at 20 fields per well with a x20 objective lens on an Operetta high content device (Perkin Elmer). Operetta images were analyzed with a customized scheme built from image analysis functions available in Harmony software.

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Data availability
Coordinates and structure factors have been deposited in the Protein Data Bank under accession number 6MAM. Other data are available from corresponding author upon reasonable request.

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| ☐   | Eukaryotic cell lines |
| ☐   | Palaeontology |
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Methods

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| ☐   | ChiP-seq |
| ☐   | Flow cytometry |
| ☐   | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials
The cleaved Ebola virus glycoprotein used in this study is available from the authors upon reasonable request.

Antibodies

Antibodies used
ADi-5946 is a human IgG antibody that was recombinantly expressed both as a Fab as well as an IgG. FVM09 is a Cynomolgus
macaque antibody and was recombinantly expressed as an IgG. A goat anti-mouse IgG-HRP from Santa Cruz Biotechnologies (sc-2005) was used as an ELISA secondary.

Validation
This is a structural biology paper. The antibody was complexed with the Ebola virus glycoprotein and this structure was solved by x-ray crystallography.

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Data deposition
☐ Confirm that both raw and final processed data have been deposited in a public database such as GEO.
☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links
May remain private before publication.
For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, provide a link to the deposited data.

Files in database submission
Provide a list of all files available in the database submission.
Methodology

Replicates
Describe the experimental replicates, specifying number, type and replicate agreement.

Sequencing depth
Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

Antibodies
Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

Peak calling parameters
Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

Data quality
Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

Software
Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

Flow Cytometry

Plots
Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.

Instrument
Identify the instrument used for data collection, specifying make and model number.

Software
Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details.

Cell population abundance
Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.

Gating strategy
Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between “positive” and “negative” staining cell populations are defined.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type
Indicate task or resting state; event-related or block design.

Design specifications
Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.

Behavioral performance measures
State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).

Acquisition

Imaging type(s)
Specify: functional, structural, diffusion, perfusion.

Field strength
Specify in Tesla

Sequence & imaging parameters
Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size,
| **Sequence & imaging parameters** | slice thickness, orientation and TE/TR/flip angle. |
|----------------------------------|--------------------------------------------------|
| **Area of acquisition** | State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined. |
| **Diffusion MRI** | Used | Not used |

**Preprocessing**

| **Preprocessing software** | Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.). |
|----------------------------|--------------------------------------------------|
| **Normalization** | If data were normalized/standardized, describe the approach(es); specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization. |
| **Normalization template** | Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI152, ICBM152) OR indicate that the data were not normalized. |
| **Noise and artifact removal** | Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration). |
| **Volume censoring** | Define your software and/or method and criteria for volume censoring, and state the extent of such censoring. |

**Statistical modeling & inference**

| **Model type and settings** | Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation). |
|-----------------------------|--------------------------------------------------|
| **Effect(s) tested** | Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used. |

Specify type of analysis: □ Whole brain □ ROI-based □ Both

**Statistic type for inference** (See Flourentzou et al. 2016)

Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.

**Correction**

Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).

**Models & analysis**

| n/a | Involved in the study |
|-----|-----------------------|
| □  | Functional and/or effective connectivity |
| □  | Graph analysis |
| □  | Multivariate modeling or predictive analysis |

**Functional and/or effective connectivity**

Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).

**Graph analysis**

Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).

**Multivariate modeling and predictive analysis**

Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.