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Structure of the Inmazeb cocktail and resistance to Ebola virus escape

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In brief
Rayaprolu et al. combine structural and functional analysis to reveal the molecular details of the Inmazeb antibody cocktail, the first FDA-approved therapeutic effective against Ebola virus disease. They show that targeting non-overlapping epitopes of pathogens is an effective strategy to combat escape mutations and mitigate the risk of drug-induced viral resistance.

Highlights
- Inmazeb antibody cocktail targets three discrete epitopes on Ebola virus GP trimer
- High-resolution structure maps in detail the three contact sites
- Inmazeb-GP structure defines GP glycan cap regions
- Unlike monotherapies, Inmazeb cocktail prevents rapid selection of drug-resistant virus
**Structure of the Inmazeb cocktail and resistance to Ebola virus escape**

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**SUMMARY**

Monoclonal antibodies can provide important pre- or post-exposure protection against infectious disease for those not yet vaccinated or in individuals that fail to mount a protective immune response after vaccination. Inmazeb (REGN-EB3), a three-antibody cocktail against Ebola virus, lessened disease and improved survival in a controlled trial. Here, we present the cryo-EM structure at 3.1 Å of the Ebola virus glycoprotein, determined without symmetry averaging, in a simultaneous complex with the antibodies in the Inmazeb cocktail. This structure allows the modeling of previously disordered portions of the glycoprotein glycans cap, maps the non-overlapping epitopes of Inmazeb, and illuminates the basis for complementary activities and residues critical for resistance to escape by these and other clinically relevant antibodies. We further provide direct evidence that Inmazeb protects against the rapid emergence of escape mutants, whereas monotherapies even against conserved epitopes do not, supporting the benefit of a cocktail versus a monotherapy approach.

**INTRODUCTION**

Nearly 40 outbreaks of Ebola virus disease (EVD) have occurred, including sustained outbreaks in 2014–2016 of over 28,000 cases and 11,000 deaths, another in 2018 involving 3,300 cases and 2,200 deaths, and a re-emergence in June 2020 that may be linked to recrudescence from a survivor of an earlier outbreak.1 Although an effective vaccine against Ebola virus (EBOV) is available, there are challenges associated with widespread vaccination and risk of breakthrough cases in vaccinated individuals.2 Given the exceptionally high mortality rate, monoclonal antibody (mAb)-based therapeutics remain the most effective treatment for patients infected with EBOV. Although several mAb therapies are in development to treat EBOV infections, REGN-EB3 (Inmazeb) is the first Food and Drug Administration (FDA) approved therapeutic for the treatment of EBOV infection, followed by mAb114 (Ansvumivab) the second.3

Therapeutic antibodies against EBOV target the viral glycoprotein (GP). EBOV GP is the only viral protein expressed on the virion surface and is required for host cell attachment, endosomal entry, and membrane fusion. In the producer cell, the GP precursor is cleaved by host furin to yield GP1 and GP2 subunits that remain linked by a disulfide bond.4 Three GP1-GP2 heterodimers associate and are displayed as trimers on the virus surface.5 The GP1 subunit drives attachment to target cells and includes the receptor-binding site (RBS), a glycan cap that shields the RBS, and a mucin-like domain (MLD). The GP2 subunit includes a transmembrane (TM) domain that anchors GP in the viral membrane, a stalk, and an internal fusion loop (IFL) that promotes the fusion of the virus with target cell membranes. After macropinocytosis-driven internalization of virions into target cells, endosomal cathepsins process GP and remove the glycan cap and MLD to yield a cleaved version of GP, termed GPCL, which can bind the intracellular receptor Niemann-Pick C1 (NPC1).5 Following receptor
Figure 1. Structural overview of REGN-EB3 bound to EBOV GP

(A) Schematic representation of EBOV GP ectodomain. The mucin-like domain (MLD) and transmembrane (TM) domains are deleted. Thermolysin (ThL) and furin cleavage sites are indicated. A disulfide bond links the N-terminal portion of GP1 (light gray) to GP2 (dark gray). The glycan cap, internal fusion loop (IFL), and heptad repeats 1 (HR1) are colored cyan, purple, and yellow, respectively. Hash-marked regions in the schematic correspond to disordered regions in the structure.

(B) Surface representation of side and top views of the EBOV GP trimer in the complex. The dotted line indicates the approximate region of the MLD. The β1-β2 loop (pink), receptor-binding site (RBS; brick red), ThL cut site (red), glycan cap (cyan), mucin-like domain (MLD; white), internal fusion loop (IFL; purple), heptad repeat 1 (HR1; yellow), and glycans (green) are shown.

(C) Surface representation of EBOV GP trimer with each of the three different antibody footprints illustrated as a black outline in three separate views The dotted line and the red triangle indicated the 3-fold axis of the GP trimer.
binding, GP2 rearranges into a six-helix bundle that drives membrane fusion. In addition to membrane-bound GP, infected cells also produce secreted GP (sGP) that carries the N-terminal 295 amino acids of GP but lacks both the GP2 and MLD. sGP is the primary product of the GP gene and may elicit production of non-neutralizing antibodies that could dampen the effectiveness of the immune response, although its function remains unclear.

Antibodies against GP have been shown to target sites across the entire molecule. Epitopes are found in the membrane-proximal stalk domain, the GP2 fusion loop, the base that includes regions of both GP1 and GP2, and the receptor-binding head, glycan cap, and MLD of GP1. Multiple studies, including a parallel analysis by an international consortium, have determined that a combination of antibody functions correlates with in vivo protection, including both neutralization (measured by blocking of infection in cell culture) and induction of multiple immune “effector” functions by the antibody Fc. Some antibodies such as those that bind the GP1 head domain achieve both neutralization and Fc-mediated activities. The combination of neutralization and effector functions are also achieved by combining complementary antibodies, for example, in the ZMapp and rEBOV-520 plus rEBOV-548 cocktails.

The REGN-EB3 (Imnazebo) cocktail contains three fully human mAbs that are each directed against a unique, non-overlapping epitope on EBOV GP. The three antibodies in the REGN-EB3 cocktail were selected based on their ability to simultaneously bind EBOV GP and on their complementary combination of functional properties. REGN3479 (maftivimab) targets the fusion loop and is potently neutralizing. REGN3471 (odesivimab) targets the GP1 head and sGP. Although REGN3471 is poorly neutralizing, it mediates effector function and is partially protective in a guinea pig model of EBOV infection. REGN3470 (atoltivimab) targets the glycan cap, is partially neutralizing, and also mediates Fc effector functions that can promote the killing of EBOV-infected cells. During the selection and development of these antibodies, it was hypothesized that a three-antibody cocktail may also reduce the potential for selection of antibody resistance by requiring the simultaneous selection of escape mutations in the GP to each component.

The therapeutic efficacy of the REGN-EB3 cocktail was evaluated in the PALM trial (Pamoja Tulinde Maisha—“Together We Save Lives”)—a randomized, controlled trial conducted in the Democratic Republic of the Congo (DRC) during the 2018 EBOV outbreak. This trial compared the efficacy of the triple mAb cocktail ZMapp (control group) with the antiviral agent Remdesivir, the single antibody mAb114, and the REGN-EB3 triple mAb cocktail. The study endpoint was survival at 28 days post-treatment. Patients treated with REGN-EB3 or mAb114 were substantially less likely to die than those treated with ZMapp (percentage surviving: 33.5% and 35.1% death, respectively, vs. 51.3%). In fact, the significantly higher efficacy of REGN-EB3 over ZMapp prompted the early termination of the clinical trial after reviewing 499 of the 700 patients enrolled. These results provided the first clinically evaluated, specific treatment options for EBOV infection. Based on the PALM study results, REGN-EB3 was granted FDA approval as the first antiviral mAb cocktail for the treatment of EVD, the first mAb product for the treatment of a viral infection, and only the second mAb product against a viral antigen.

The lack of high-resolution structure for the REGN-EB3 cocktail in complex with EBOV GP complicates the understanding of the precise contacts that mediate its functions and its complementary activities. Mapping the epitope footprints of these three antibodies can facilitate the monitoring of clinical isolates for GP that carry mutations that are resistant to REGN-EB3. Indeed, the ability of EBOV GP to acquire mutations and develop drug-induced resistance to approved mAb therapeutics has not been previously evaluated. The relevance of in vitro viral escape studies with SARS-CoV-2 has now been confirmed in the clinical setting, with the demonstration that monotherapy treatment can select drug-resistant viruses in treated individuals.

Here, we report a 3.1 Å resolution cryo-EM structure of the complete REGN-EB3 cocktail simultaneously bound to the EBOV GP trimer. This structure maps the non-overlapping epitopes of REGN-EB3 and illuminates the basis for complementary activities, as well as residues that are critical for resistance to escape by each component of this cocktail and other clinically relevant antibodies. We also provide direct evidence that, unlike monotherapy treatments, including those targeting conserved epitopes, the REGN-EB3 combination protects against the rapid emergence of EBOV escape mutants and supports the benefit of the combination approach, as has been extensively demonstrated for SARS-CoV-2.

RESULTS

To define the structural basis of binding, neutralization, and protection by the REGN-EB3 cocktail of antibodies, we first carried out the cryo-EM analysis of Fabs of the REGN-EB3 cocktail in complex with recombinant, fully glycosylated EBOV GP that lacks the MLD (Mayinga strain, Figure 1A). In initial 2D class averages, complexes were mostly fully occupied with nine Fab fragments (i.e., three copies of each of the three different antibodies). In subsequent 3D classification, the occasional partial occupancy resulted in some classes having nine Fab fragments bound, and others having eight Fab fragments bound (two copies of REGN3470, plus three each of REGN3471 and REGN3479). Homogeneous and non-uniform refinements using Cryo-EM Single Particle Ab-Initio Reconstruction and Classification (cryoSPARC) yielded a cryo-EM reconstruction of the complex resolved to ~3.1 Å. Obtaining a high-resolution map was only possible when particles from both occupancy classes were used for subsequent refinements. Models of the GP and the Fab Fv only were docked into the density and refined (Figures 1B, 1C, S1, and S2; Tables S1 and S2). We determined this structure using C1 symmetry so that individual differences

See also Figures S1, S2, and S6 and Tables S1 and S2.
among the monomers can be discerned. All previous cryo-EM structures of EBOV GP at this resolution were determined using C3 symmetry in which the three monomers in the trimer were averaged around the 3-fold axis. There is typically a trade-off between resolution and examination of asymmetry: resolution is improved by averaging monomers together, but biologically relevant individual asymmetric elements are lost during the computational process. For this structure, the application of C3 symmetry and averaging resulted in a loss of signal for all three Fv regions of REGN3470 to the extent that precluded unambiguous model building. By contrast, the C1 symmetry maps revealed clear density for one copy of REGN3470 and moderately strong density for a second copy, which allowed us to build two copies of this antibody into the final structure.

REGN3479 Fab recognizes a quaternary epitope

The REGN3479 Fab binds a quaternary epitope that bridges the paddle-shaped, hydrophobic fusion loop of one GP monomer, monomer A, to a GP1/GP2-containing site in an adjacent monomer, monomer B (Figure 2A). Within this footprint, REGN3479 binds residues 527–530, 535, and 536 of the fusion loop of monomer A, as well as a conformational site involving three polypeptide sections of monomer B and a glycan. This conformational site comprises GP1 residues Pro34, Leu43, and Val529 at the tip of the fusion loop was previously shown to bend inward to form a hydrophobic bridge with Ile544. Phe535 interacts with this bridge to form a hydrophobic patch that is required for the formation of a fusion-competent fist-like structure. Phe32 in the REGN3479 light chain (LC) disrupts the formation of this hydrophobic patch formation by wedging between Leu529 and Phe535 of monomer A in the GP trimer (Figure 2C). REGN3479 binding introduces other conformational adjustments on GP as well including a ~7 Å shift mediated by REGN3479 binding.

See also Table S3.
rotation of Ile527 in the fusion loop paddle into a hydrophobic pocket created by the heavy-chain (HC) Trp47 and LC Tyr59. Furthermore, residues 549–553 of the fusion loop stem C terminus translate, and residues 552–553 lift to contribute to a helical pocket created by the heavy-chain (HC) Trp47 and LC Tyr59. The side-chain atoms of LC residues Tyr49 and Gln55 and the side-chain atoms of Lys98, Arg97, respectively, as well as the side-chain atoms of Tyr31 and Gly52 Asp56 form hydrogen bonds with GP Glu112. Meanwhile, the CDRL2 in REGN3471 Fab displace the GP Thr270 by ~9 Å into the RBS to form high-affinity salt bridges and also participate in other interactions.

REGN3471 stabilizes the β17/β18 loop in the glycan cap

REGN3470 binds to the top of the glycan cap of GP1 (Figure 1C), at a site involving the antiparallel β17 and β18 strands, which are connected by a 27-residue descending loop (residues 279–306) (Figure 2A). Notably, this structure of the GP-REGN3470 complex allows visualization of the entire β17/β18 loop (Figure 4A), which was disordered in all previous structures of EBOV GP. In complex with the REGN3470 Fab, the β17/β18 loop of GP is fully ordered, and the main-chain direction as well as the identity and position of the side chains are clear. Interestingly, this clarity occurs only for the two GP monomers that are in complex with the REGN3470 Fab. In the remaining unbound GP monomer, residues 262–270, 280–282, and 293–311 are disordered, similar to previous GP structures.

The β17 and β18 strands of the GP glycan cap are bound by 19 residues of REGN3470, 10 of which interact with Asn278, Glu280, and Asp282 (Table S3). The increased order of the loop connecting the β17 and β18 strands in the REGN-EB3 complex likely arises from the binding of bulky and polar antibody residues in the antibody HC including Asn31, Tyr32, His53, and Asn99. This binding consequentially drives GP residues 277–280 of the β17 strand ~6 Å inward toward the base of GP helix α2. The ordering of the loop creates a new intra-GP backbone interaction between Ile285 and Arg247 that causes the intervening GP residues 281–285 to buckle outward, bringing the entire loop encompassing residues 285–312 into order.
We also treated pre-formed individual GP-Fab complexes with ThL and monitored the cleavage event over time using western blotting under non-reducing conditions. Digestion of unbound GP with ThL over time resulted in the disappearance of the major intact GP band (~67 kDa) and the appearance of the major digestion product, GPCL (~37 kDa), as well as an intermediate (~50 kDa) band. Interestingly, ThL digestion of REGN3479 complexed with GP showed a clear reduction in GPCL band intensity and a small increase in the intensity of the larger intermediate (~50 kDa). The marked reduction in GPCL band intensity suggests that the presence of REGN3479 may block the ThL cleavage site (Figure 5B). No discernible difference in cleavage patterns was seen for GP complexed with either REGN3470 or REGN3471 compared with unbound GP (data not shown).

We further evaluated the formation of a stable GPCL-antibody complex using size-exclusion chromatography (SEC). GPCL was incubated with each of the Fabs in the REGN-EB3 cocktail individually, and the elution profile of the complex was monitored using SEC (Figure S4A). GP cleavage did not affect complex formation with REGN3479 as evidenced by the presence of two peaks in the SEC trace, one corresponding to the complex and the other to free Fab. REGN3470 for which the epitope lies exclusively in the glycan cap does not bind cap-deleted GP. Only peaks corresponding to only GPCL and free Fab were observed—with no peak for the complex. REGN3471 for which the glycan cap comprises ~40% of the epitope exhibited some binding of GPCL—three peaks, corresponding to complex, GPCL, and free Fab are observed (Figure S4A).

**REGN-EB3 prevents rapid viral escape**

We used a chimeric Vesicular stomatitis virus (VSV) expressing EBOV GP (VSV-EBOV-GP) to characterize the likelihood of escape mutations in GP following exposure to individual mAbs and to competing and non-competing antibody combinations. As a first step, we tested the neutralization potency of these antibodies against VSV-EBOV-GP (Figures 6A and 6B). Consistent with previous reports, REGN3479 and mAb114 were potently neutralizing in our assay, with IC50s of...
to the contact residues of REGN3470, REGN3471, and REGN3479 (Figure 6E). Mutations that were previously shown to impact antibody neutralization were also selected for other antibodies against EBOV GP including KZ2 (G557R), 2G4 (Q508R), and 4G7 (G557R) mAbs.

To understand how epitope conservation correlates with the development of antibody-induced resistance, we assessed the escape kinetics of mAb114 and REGN3479. mAb114 binds to a highly conserved region in the receptor-binding domain (RBD) required for viral entry,

while REGN3479 binds the conserved fusion loop and can neutralize multiple ebolaviruses, including EBOV Mayinga, EBOV Mali, Bundibugyo virus, and Sudan virus (Figure S4B). Both mAb114 and REGN3479 rapidly selected resistant viruses within a single passage, similar to all other individual antibodies (Figures 6D and 6E). Furthermore, viruses resistant to mAb114 could be plaque purified and expanded without reversion of the escape mutations or obvious fitness defects.

Next, we assessed the evolution of resistance to antibody cocktails containing components targeting overlapping (ZMapp) or distinct epitopes (REGN-EB3 and REGN3479 + mAb114). Similar to the monotherapies, the selection of a single variant (Q508R) led to complete escape from the triple ZMapp combination containing two neutralizing antibodies that bind overlapping epitopes (2G4 and 4G7; both of which are affected by Q508R) plus one non-neutralizing antibody (13C6). Q508R was also identified in an EBOV-infected cynomolgus macaque that succumbed to the virus after ZMapp therapy and led to resistance to the cocktail, confirming that similar mutations are selected in vitro with the pseudotyped virus in vivo with authentic EBOV.

However, combinations of three (REGN-EB3) or two (REGN3479 + mAb114) antibodies directed against distinct epitopes increased resistance to viral escape. Ten passages and multiple mutations were required to escape the potently neutralizing two-mAb combination (REGN3479 + mAb114). The emergence of complete resistance was not observed with REGN-EB3 following 10 passages (Figure 6E).

To validate the mutants detected through sequencing analysis, we generated lentivirus-based pseudoparticles (EBOVpp) bearing individual escape mutations within the 2014 Zaire EBOV GP sequence. As expected, the neutralization potency of every individual neutralizing mAb was impacted by mutations detected during selection (Table 1; Figure S5). Two identified mutants (P34S and T469I) did not impact antibody potency. These were likely tissue culture adaptations or were genetically linked to other variants that affect antibody neutralization selected during passaging. Despite the conservation of the mAb114 epitope, four mutants isolated under either mAb114 monotherapy or REGN3479 + mAb114 cocktail selection were infectious and resulted in the complete loss of mAb114 neutralization activity. A single mutation (Q508R), also identified in previous studies, was sufficient for complete resistance to ZMapp and the two competing individual neutralizing components (2G4 and 4G7). Importantly, no single individual escape mutation impacted the neutralization of the non-competing three mAb (REGN-EB3) or two mAb (mAb114 + REGN3479) combinations that target distinct and non-overlapping antibody epitopes.

Figure 5. Glycan cap removal abolishes REGN3470 GP binding and reduces REGN3471 GP binding
(A) Ligand binding properties of REGN-EB3 cocktail antibodies. Summary of equilibrium dissociation constants (K_D) for the interaction of surface-captured anti-EBOV antibody with recombinant EBOV GP or GPCL trimer protein, respectively. k_a, association rate constant; k_d, dissociation rate constant; K_D, equilibrium dissociation constant; t_1/2, dissociative half-life.

(B) Time-dependent thermolysin cleavage of GP in the absence and presence of REGN3479. See Figures S3 and S4.

25.9 and 54.7 ng/mL, respectively. REGN3470 also demonstrated high neutralization potency, although neutralization never reached 100%, leaving a small population of infectious virus un-neutralized even at high antibody concentrations (Figure 6C), as previously observed. REGN3471 was poorly neutralizing. The non-competing REGN-EB3 and REGN3479 + mAb114 combinations also potently neutralized VSV-EBOV-GP.

To determine the likelihood of mAb-induced viral escape and identify the mutations in GP associated with loss of therapeutic neutralizing activity, escape studies with VSV-EBOV-G were conducted by passaging the virus in the presence of either individual mAbs or mAbs in combination. Notably, virus resistance characterized by cytopathic cell death was observed within one or two passages for all mAbs used as a monotherapy (Figure 6D). Sequencing analysis revealed that single mutations in the GP protein arising in these one or two passages could result in complete viral resistance to all individual neutralizing mAbs (Figure 6E). Notably, mutations were selected within or adjacent
Figure 6. REGN-EB3 prevents rapid viral escape

(A) Neutralization curves of fully replicative VSV-EBOV-GP by individual REGN-EB3 components, mAb114, a two-mAb combination (REGN3479 + mAb114), or the three-mAb REGN-EB3.

(B) Table showing IC50 values for VSV-EBOV-GP neutralization by different antibodies.

(C) Graph showing the neutralization fraction of different antibodies at various concentrations.

(D) Table showing the passage and viral replication status for different antibody concentrations.

(E) Table showing the antibody selection and passage results for different variants.

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DISCUSSION

The recent clinical success of mAb therapies for EBOV and SARS-CoV-2 clearly demonstrates that antibody therapeutics targeting viral proteins offer a valuable option for both treatment and prophylaxis of viral infections. 1-3,10 Although vaccination will always be a critical component of the public health response to infectious agents, it is clear that a significant portion of the population will remain vulnerable to severe outcomes due to suboptimal responses to vaccines or challenges associated with vaccine access or uptake. Mass vaccination campaigns for infectious agents like EBOV may never occur, necessitating the availability of therapeutics in the event of disease outbreaks that are unpredictable in timing and location. Antibody-based therapeutics may offer additional protection from severe disease and death even to vaccinated individuals that fall into high-risk categories. Thus, antibody therapies that can be rapidly deployed can provide immediate treatment and protect recipients until vaccination and its associated immune response can mount. Indeed, 25% of PALM trial participants who experienced some level of EBOV disease reported that they had been vaccinated before the onset of symptoms. 2

Protection against EBOV infection by an antibody cocktail can be achieved by combining complementary activities, including both neutralizing and Fc-recruiting functions. 9,10 REGN-EB3 combines individual mAbs with neutralizing (REGN3479 and REGN3470) and Fc-recruiting (REGN3470 and REGN3471) functions. Importantly, this cocktail contains antibodies with non-overlapping footprints; the distinction of the contact residues limits the likelihood of treatment-induced drug resistance.

Here, we present the cryo-EM structure at 3.1 Å resolution of the complete REGN3470, REGN3471, and REGN3479 cocktail bound to EBOV GP. Asymmetric reconstruction revealed eight Fab fragments simultaneously bound to the GP trimer: three each of REGN3470 and REGN3471 and two copies of REGN3470 in the final high-resolution model. We are not aware of any published EBOV GP structure bound to antibodies that have been resolved to this resolution or better using asymmetric reconstruction of cryo-EM data. The ordering of this ~600 kDa complex and the binding of the REGN3470 antibody contained within it allow modeling key portions of the glycan cap, including the β17-β18 strands and the 27-residue loop in between. These sections were either fully disordered or only visible as main-chain tubes of indiscernible direction in previous crystal or cryo-EM structures. Here, they have been built in entirety, with visible side chains to confirm sequence and chain directionality. This model now corrects the direction of the chain in a previous high-resolution model in which density in this region was still unclear.

This cryo-EM structure further reveals the contents and key contacts contained within the three non-overlapping footprints of the component members of the cocktail. REGN3479 binds to a quaternary epitope bridging the fusion loop tip and paddle of one GP monomer to both GP1 and GP2 of the neighboring monomer. One key mechanism of action is by locking the GP in the pre-fusion conformation, similar to other fusion loop binding antibodies like ADI-15878, 20 and indeed, the epitope recognized by REGN3479 almost completely overlaps with that of ADI-15878. Ten of the 15 GP residues that interact with REGN3479 are shared with ADI-15878, and both antibodies recognize the conserved N-terminal pocket while displacing the non-conserved N terminus of GP2. However, only REGN3479 also contacts GP2 HR1 α. A second difference is that REGN3479 binds only the glycan itself, whereas ADI-15878 also binds the Asn side chain to which the glycan is linked.

Antibodies binding overlapping epitopes can have very different neutralization potentials that are hard to reconcile without high-resolution structures. For example, the general footprint and the overall vertical position of non-neutralizing REGN3471 resemble those of mAb114, 26 a potently neutralizing therapeutic antibody used as a monotherapy. A patch of hydrophobic residues in REGN3471 binds into the RBS underneath the hydrophilic crest of the GP (Figure 3D, left). By contrast, in mAb114, two arginines [Arg30 and Arg97] potentially form strong salt-bridging interactions with the GP crest residues Glu231 and Glu235, respectively (Figure 3D, right). Further, REGN3471 interacts with eight residues of the glycan cap while mAb114 interacts with only four. The greater dependence on glycan cap residues for binding likely explains why mAb114 exhibits higher affinity binding than REGN3471 to EBOV-GPctl and greater neutralization potency. The similarity of footprints and HCUs utilized by these two antibodies demonstrates that Velocimmune mice can generate nearly identical antibody responses to humans. 10,36

Fc-mediated immune effector functions play an important role in in vivo protection. 3 The existence and strength of these functions are controlled by antibody binding angle and accessibility of the Fc region to the respective Fc receptors. Although each of the antibodies in the REGN-EB3 cocktail have identical Fc domains, only REGN3470 and REGN3471 show potent immune effector functions—REGN3479, which binds to the side instead of the top of GP, has none. 13 REGN3470 binds radially outward from the top of the GP structure, anchoring solely to the glycan cap. REGN3471 binds vertically upward, anchoring into the RBS of EBOV GP and bridging it to the glycan cap. When homology models of REGN3470, REGN3471, and REGN3479 IgGs and a crystal structure of the receptor FcγR-IIIa bound to an IgG are docked into the cryo-EM map, it is evident that the Fc
regions of REGN3470 and REGN3471 are easily accessible for FcγR-IIIa receptor binding while the Fc region of REGN3479 has limited accessibility (Figure S6A).

A key desired characteristic of any antiviral therapy is the minimal likelihood of drug-induced resistance, both in the treated individual and in the population in which a mutant virus may spread. With antibody therapeutics, these concerns may be especially great as mAb-induced mutations may also impact vaccine efficacy since these mutations may occur in epitopes recognized by endogenous polyclonal antibody responses as well. Although the risk of drug resistance cannot be fully eliminated, it can be greatly reduced with combination approaches. We have previously assessed the generation of viral resistance to SARS-CoV-2 spike antibody monotherapies and combinations and demonstrated that resistant viruses were rapidly selected with monotherapies and overlapping-epitope combinations, regardless of epitope conservation.16,18 By contrast, the use of antibody combinations binding distinct epitopes reduced the risk of rapid viral escape by requiring concurrent selection of resistance mutations to each antibody.37,38 Importantly, these findings based on surrogate in vitro escape studies were subsequently confirmed in real-world clinical data in COVID patients, validating the surrogate system as a relevant predictor of clinical resistance risk.37,38

In summary, the combined structural and functional analysis here reveals the footprints and key contacts of an FDA-approved therapeutic effective against EVD. It also demonstrates that cocktails of antibodies targeting non-overlapping epitopes are key to mitigating the risk of drug-induced viral resistance.

**STAR+METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
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  - Viruses
- **METHOD DETAILS**
  - GP Expression and purification
  - Cleavage and purification of REGN3470, REGN3471 and REGN3479 Fab fragments

| Table 1. Fold-decrease in potency relative to wild-type (WT) pseudovirus |
|---------------------------|-------------------|-------------------|-------------------|-------------------|
| REGN3479 | REGN-EB3 | mAb114 | REGN3479 + mAb114 | 2G4 | 4G7 | ZMapp | KZ52 |
| WT | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| P34S | 0.6 | 0.4 | 0.4 | 0.5 | 0.7 | 0.6 | 0.6 | 0.5 |
| P116H | 0.6 | 0.6 | >76.3a | 0.8 | 0.7 | 0.7 | 0.9 | 0.6 |
| G118E | 0.5 | 0.4 | >29.1a | 0.5 | 0.9 | 0.7 | 0.7 | 0.5 |
| G118R | 0.6 | 0.5 | >33.6a | 0.6 | 0.9 | 0.7 | 0.8 | 0.6 |
| T144M | 1 | 1 | >33.6a | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 |
| G224D | 0.6 | 0.6 | 1.3 | 0.8 | 0.7 | 0.7 | 0.7 | 0.7 |
| E304A | 0.7 | 0.7 | 0.5 | 0.6 | 0.3 | 0.2 | 0.4 | 0.4 |
| T469I | 0.8 | 0.7 | 0.6 | 0.7 | 0.9 | 0.7 | 0.6 | 0.7 |
| Q508R | 2.9 | 3.1 | 1.9 | 2.9 | >88.3a | >77.3a | >73.5a | >69.5a |
| G528R | >361.5a | 1.9 | 0.9 | 4 | 0.4 | 0.3 | 0.9 | 0.8 |
| G557R | 0.9 | 0.7 | 0.5 | 1 | 0.8 | >74.5a | 1.3 | >77.1a |
| N563Y | >424.0a | 0.9 | 0.4 | 3 | 0.5 | >173.4a | >162.1a | >151.7a |

The change in mAb potency for this variant was calculated by dividing the maximal antibody concentration tested by the IC50 value for the WT pseudovirus. REGN3470, REGN3471, and 13C6 poorly neutralize lentivirus pseudovirus particles and could not be tested with escape variants. See also Figures S4 and S5.
REGN3471 crystal screening, data collection and structure determination
Preparation of GP-REGN-EB3 complex
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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chom.2023.01.022.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The Regeneron employees have stock and/or options in the company. C.A.K. is an officer of the company. The antibodies are patented: WO/2016/123019.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as a gender minority in their field of research. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

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REFERENCES


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# STAR METHODS

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| **Bacterial and Virus Strains** |        |            |
| VSV-EBOV-GP                  | This manuscript | N/A        |
| EBOV Mayinga                 | Rocky Mountain Laboratories, Dr. Heinz Feldmann | N/A        |
| EBOV Mali                     | Rocky Mountain Laboratories, Dr. Heinz Feldmann | N/A        |
| RESTV                         | Rocky Mountain Laboratories, Dr. Heinz Feldmann | N/A        |
| BDBV                          | Rocky Mountain Laboratories, Dr. Heinz Feldmann | N/A        |
| SUDV                          | Rocky Mountain Laboratories, Dr. Heinz Feldmann | N/A        |
| MARV Musoke                   | Rocky Mountain Laboratories, Dr. Heinz Feldmann | N/A        |
| MARV Angola                   | Rocky Mountain Laboratories, Dr. Heinz Feldmann | N/A        |

| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Papain                           | Sigma | Cat# P3125 |
| Iodoacetamide                    | Sigma | Cat# I6125 |
| BioLock                          | IBA Lifesciences | Cat# 2-0205-250 |
| Lauryl maltose neopentyl glycol  | Anatrace | NG310    |
| Ethanolamine                     | Cyntiva | Cat# BR100050 |
| Thermolysin L                    | Sigma | P1512-25mg |
| TRIzol Reagent                   | Life Technologies | Cat# 15996 |
| Insect-XPRESS protein-free medium with L-glutamine | Lonza | 12-730Q |
| Puromycin                        | Invivogen | ant-pr-5b |

| **Critical Commercial Assays** |        |            |
| 4D-Nucleofector X Kit L         | Lonza | Cat# V4XC-2012 |
| BrightGlo luciferase assay      | Promega | E2610  |
| Phosphate-buffered saline       | Life Technologies | 20012-043 |
| Sucrose                          | Invitrogen | 15503-022 |

| **Deposited Data**               |        |            |
| Structure of EBOV GP (Mayinga) bound to three REGN 3471 fabs, two REGN 3470 fabs, and three REGN 3479 fabs | Protein Data Bank ([rcsb.org](https://www.rcsb.org)) | PDB: 7TN9 |

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## REAGENT or RESOURCE SOURCE IDENTIFIER

### Cryo-EM map of EBOV GP (Mayinga) bound to three REGN 3471 fabs, two REGN 3470 fabs, and three REGN 3479 fabs

Cryo-EM map of EBOV GP (Mayinga) bound to three REGN 3471 fabs, two REGN 3470 fabs, and three REGN 3479 fabs

Electron Microscopy Data Bank (https://www.ebi.ac.uk/emdb/)

EMD-26005

### Experimental Models: Cell Lines

| HEK293T | ATCC | CRL-3216 |
| Vero E6 | ATCC | CCL-81 |
| BHK-21  | ATCC | CCL-10 |
| Drosophila S2 | Thermo Fisher Scientific | Cat# R69007 |
| Huh7    | JCRB Cell Bank | JCRB0403 |

### Recombinant DNA

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### Software and Algorithms

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RESOURCE AVAILABILITY

Lead Contact
Further information requests should be directed to the lead contact Erica Ollmann Saphire (erica@lji.org).

Materials availability
All reagent requests in this study should be directed to the lead contact Erica Ollmann Saphire (erica@lji.org). Requests for REGN3479, REGN3471 and REGN3470 should be directed to Christos Kyratsous (christos.kyratsous@regeneron.com).

Data and code availability
The cryo-EM map has been deposited at the Electron Microscopy Data Bank (www.ebi.ac.uk/emdb). The accession number for the map is EMD-26005. The atomic model corresponding to the microscopy data has been deposited in the Protein Data Bank under the accession number PDB: 7TN9. No custom code was generated as part of the study.
Any additional information required to reanalyze the data reported in this work paper is available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
HEK293T, Vero, Huh7, and BHK-21 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies), supplemented with 10% by volume heat inactivated Fetal Bovine Serum (FBS, Life Technologies), and 1% by volume Penicillin-Streptomycin-Glutamine (PSG, Life Technologies).

Drosophila Schneider S2 cells were grown in Schneider’s Drosophila Medium at 27°C to generate stable cell lines. Insect-XPRESS protein-free medium with L-glutamine (Lonza) supplemented with puromycin (6 μg/mL) (InvivoGen) was used to grow S2 cells at 27°C for protein expression.

Viruses
The cloning and rescue of VSV-EBOV-GP is described below. Viral stocks were produced by infecting sub-confluent monolayers of BHK-21 at a multiplicity of infection (MOI) of 0.01. At 16-24 hours post infection, supernatants were collected and spun at 3000xg to remove cellular debris. Viral stocks were concentrated 25-fold by sucrose cushion ultracentrifugation, resuspended in Phosphate-buffered saline (PBS, Life Technologies), and frozen at -80°C. Viral stocks were then titered by plaque assay on BHK-21 cells and sequence-confirmed by RNAseq (described below).

METHOD DETAILS

GP Expression and purification
Both mucin-containing and mucin-deleted EBOV GP were produced from stably transfected Drosophila melanogaster S2 cells. Briefly, Effectene (Qiagen) was used to transfect S2 cells with a modified pMT-puro vector plasmid containing the GP gene of interest. Transfected cells were selected by incubation at 27°C for 4 weeks in complete Schneider’s medium with 6μg/mL puromycin. The cells were then transitioned to Insect Xpress medium (Lonza) for large-scale expression in 2-liter Erlenmeyer flasks. Expression of secreted GP ectodomain was induced with 500 mM CuSO4, and the supernatant was harvested after 5 days. The GP was engineered with a double Strep-tag at the C terminus to facilitate purification using a 5 mL Strep-trap HP column (GE) and then further purified by Superdex 200 size exclusion chromatography (SEC) in 50mM Tris-buffered saline (Tris-HCl, pH 7.5, 150mM NaCl [TBS]).

Cleavage and purification of REGN3470, REGN3471 and REGN3479 Fab fragments
To produce Fab fragments, each antibody (10mg) was digested with 200μg papain (5% w/w) for four hours at 37°C; the reaction was quenched with 50mM iodoacetamide. The digested protein was dialyzed overnight at 4°C against 10mM Tris pH 8.0, 10mM NaCl buffer. The Fab fraction was then purified using a MonoQ 2mL column according to the manufacturer’s protocol, followed by size exclusion chromatography using a Superdex 75 increase column equilibrated with 25mM Tris, 150mM NaCl, pH 7.5. The fractionated peak was concentrated in 10kDa MWCO Amicon Ultra 15 concentrators.

REGN3471 crystal screening, data collection and structure determination
Crystal screening of unbound Fab fragments of REGN3471 (6.6mg/mL) was performed using sparse matrix screens and an Oryx crystallization robot (Douglas instruments). Initial hits for crystals were obtained with 20% isopropanol, 20% PEG 4000, 100mM sodium citrate, pH 5.6. The crystals diffracted to 2.3Å at APS beamline 23-ID-B. The collected data were processed using XDS. Crystals were indexed in space group P2_1_2_1 and contained two Fabs in the asymmetric unit. Molecular replacement was used to iteratively determine phases. The initial molecular replacement model was generated by submitting the REGN3471 sequence to the SWISS-MODEL server. Rebuilding and refinement were performed using Phenix and Coot. Molprobity and OMIT maps were used to validate model quality (Figure S1).

Preparation of GP-REGN-EB3 complex
To obtain complexes of GP with Inmazeb, GP (120μg) and the Fab fragments of REGN3470 (135μg), REGN3471 (125μg) and REGN3479 (150μg) were incubated together at room temperature overnight in a total volume of 500μL. The resulting complex was separated from excess Fabs using a Superose 6 Increase column equilibrated with 25mM Tris, 150mM NaCl, pH 7.5. Fractions containing the complex were combined and concentrated to 3.1mg/mL using a 100kDa MWCO Amicon Ultra 15 concentrator.

Cryo-EM Sample preparation and data collection
C-flat™ EM (CF-2/1-4C-T, Electron Microscopy Sciences) grids were glow-discharged for 15 seconds on a Pelco Easiglow at 15nA power. The purified GP-REGN-EB3 complex (3μL, ~0.8mg/mL) was mixed with 1μL 0.02mM lauryl maltose neopentyl glycol (LMNG) in 1X TBS and immediately applied to glow-discharged grids at 4°C and 100% relative humidity inside a Vitrobot Mark IV. The grids were then blotted for 8 seconds without any extra applied blot force and plunge-frozen in liquid ethane. Frozen grids were imaged on a Thermo Fisher/Scientific Titan Krios (G3) equipped with a K2 direct electron detector and a BioQuantum energy filter (Gatan) using
SerialEM software. In initial efforts to freeze the GP-Fab complex onto grids, particles stuck to the grid hole edges, making data acquisition exceedingly difficult. Mixing the complex with the amphiphilic detergent Lauril Maltose Neopentyl glycol (LMNG), immediately before freezing dramatically improved the observed particle count and enabled successful data collection. Three separate datasets were collected on separate days. A total of 6,360 movies were collected at 1.0504 Å/pixel with 30 frames per movie and a dose of 33-52e⁻/Å². Defocus targets cycled from -0.8 to -2.6 microns.

**Cryo-EM map calculation, structure determination and structure refinement**

All cryo-EM data processing was performed using cryoSPARC. Patch Motion correction and Patch CTF estimation were used to align and calculate the contrast transfer function (CTF), respectively. The cryoSPARC blob picking utility was used to pick initial particles to develop a training data set of 3,154 particles that was fed into the Topaz particle picker to yield 403,095 particles. The particle set was further 2D-classified to remove irrelevant particles. The resulting particle set was used for ab-initio reconstruction. Five ab initio classes were requested and all five classes were further subjected to heterogeneous refinement using all collected particles to generate representative 3D volumes. Of the five 3D volumes, two, containing 121,135 and 155,580 particles each, showed more promising features than the others (e.g., high-resolution features, antibody occupancy, and trimeric organization). One of these two 3D volumes contained two copies of REGN3470 Fab, whereas the other contained three copies, one of which is poorly ordered. The two particle sets combined yielded a final higher resolution map that contained two copies of REGN3470 and three copies each of REGN3471 and REGN3479. Hence, although the structure shows two copies, it is clear that complete occupancy is possible in solution. These two classes were combined in homogeneous refinement followed by the final, non-uniform 3D refinement. All refinements were done with no symmetry explicitly applied (C1). The final refinement produced a 3.1Å map (using the Fourier shell correlation [FSC] = 0.143 criterion) containing 276,715 particles. This map was further improved using DeepEMshancer, a neural network-based density modification tool. Both the raw map and density-modified map were used to guide model building. A local resolution map is included as Figure S6B.

The GP-REGN-EB3 model was built by initially docking copies of the unbound REGN3471 Fab crystal structure and homology models of REGN3470 and REGN3479 generated using SWISS-MODEL and a published structure of GP (PDB 5JQ3). The EM density was clear for the Fv portions of the docked Fabs with unambiguous main-chain trace and conformation of all antibodies. Several rounds of Phenix refinement, followed by manual rebuilding using Coot, yielded the final structure. Interacting residues of GP with the Fabs were selected using LigPlot+ and were further manually validated with interatomic distances <4Å for hydrogen bonds/salt bridges or hydrophobic surfaces with interatomic distances <5Å.

Structures were visualized and analyzed using UCSF Chimera and UCSF ChimeraX. Publication figures were made using Inkscape [https://inkscape.org]

**BIAcore surface plasmon resonance analysis**

Binding kinetics and affinities for each antibody in the REGN-EB3 cocktail and for mAb114 were individually assessed using surface plasmon resonance (SPR) technology on a Biacore T200 instrument (Cytiva, Marlborough, MA) using a Series S CM5 sensor chip in filtered and degassed HBS-EP running buffer (10 mM HEPES, 150 mM NaCl, 3mM EDTA, 0.05% (v/v) polysorbate 20, pH 7.4). Capture sensor surfaces were prepared by covalently immobilizing a mouse anti-human Fc mAb (REGN2567) on the chip surface using the standard amine coupling chemistry, as reported previously. Following surface activation, the remaining active carboxyl groups on the CM5 chip surface were blocked by injecting 1M ethanolamine, pH8.0 for 7 minutes. A typical resonance unit (RU) signal of ~11,000 RU was achieved after the immobilization procedure.

Analysis of REGN3479, REGN3471, REGN3470 or mAb114 binding to EBOV-GP-ΔTM, and EBOV GP lacking the MLD (EBOV-GP-Δmuc) or glycan cap (glycan cap removed-EBOV-GP-Δmuc) was carried out by capturing the antibodies over immobilized anti-human Fc surface at 37°C. Following the capture of the antibodies, different concentrations of EBOV-GP proteins (6.25nM-200nM, two-fold serial dilution in duplicate) in the running buffer were injected for 2.5 minutes at a flow rate of 50μL/min with an 8-minute dissociation phase. At the end of each cycle, the anti-human Fc surface was regenerated using a 12-second injection of 20mM phosphoric acid.

**Thermolysin cleavage studies**

GP (2 μg) was mixed with 2.25 μg of each antibody separately and incubated at RT overnight before ~2 μg of the complex was treated with 0.5 μg Thermolysin L for 1 hour. Aliquots of the reaction were taken at indicated time points and quickly quenched with 5mM EDTA final concentration. Samples from each time point were evaluated by SDS-PAGE and western blotting.

**Size-exclusion chromatography**

GPCl (50 μg) was used to make a complex with equal amounts of each of the antibodies in REGN-EB3 individually. Mixtures were incubated for 4hrs at room temperature and run on S200i size exclusion column (Cytiva) equilibrated with 25mM Tris-base, 150mM NaCl pH 7.5 to monitor complex formation. Uncleaved GP (GP) and GPCl alone were also separately run on the S200i column as controls.

**VSV-EBOV-GP generation**

Fully replicative VSV-EBOV-GP was generated as previously described (Lawson, Rose/Whitt, Baum). In short, full-length North Kivu EBOV GP (GenBank accession MK007329.1) was cloned in place of VSV-G in a T7 promoter-driven VSV rescue plasmid. This
genomic clone and the required expression plasmids for virus rescue (VSV-N, VSV-P, VSV-L, T7 polymerase) were transfected into HEK293T cells for 48 hours. The transfected cells were then co-cultured with BHK-21 cells transfected with VSV-G using the SE cell Line 4D-Nucleofector X Kit L (Lonza) and cultured until virus-mediated CPE was observed. The recovered virus was then plaque-purified twice to isolate individual clones.

**Escape studies**

VSV-EBOV-GP studies were performed as described for VSV-SARS-CoV-2-spike with minor modifications. Briefly, 1.25 x 10⁶ pfu of VSV-EBOV-GP was incubated with serial dilutions of antibodies at room temperature for 30 minutes, and then used to infect 2.5 x 10⁵ Vero cells (ATCC: CCL-81). The cells were then monitored for virus replication (CPE) for four days. Once the majority of cells displayed CPE, the supernatants were collected and frozen at -80 °C. Total RNA was extracted using TRIzol (Life Technologies) from the cell population and subjected to RNAseq to identify variants in the EBOV-GP protein. Each subsequent passage was performed by incubating 100μL of the supernatant under the same or higher antibody concentration until resistance to ≥ 10μg/mL antibody selection was observed in at least one passage or up to 10 passages.

**Virus RNA Sequencing**

RNA was quantified using the Qubit RNA HS Assay Kit (ThermoFisher). 1 to 50 ng RNA was treated by FastSelect-rRNA HMR (Qiagen) to remove host rRNA, and the incubation condition for fragmentation was followed as 85°C for 6 minutes, 75°C for 2 minutes, 70°C for 2 minutes, 65°C for 2 minutes, 60°C for 2 minutes, 55°C for 2 minutes, 37°C for 5 minutes, 25°C for 5 minutes, and hold at 4°C. Strand-specific RNA-seq libraries were prepared from the treated RNA using KAPA RNA HyperPrep Kit (Roche Sequencing). UDI with UMI Adapters (IDT) were ligated. Sixteen-cycle PCR was performed to amplify libraries. Sequencing was run on MiSeq (Illumina) by multiplexed paired-read run with 2X70 cycles.

**VSV-EBOV-GP neutralization assays**

Neutralization assays with the fully replicative VSV-EBOV-GP were performed by incubating 2,000 pfu of virus with serial dilutions of antibody starting at a concentration of 100μg/mL. The virus/antibody mixture was incubated at room temperature for 30 minutes and used to infect 2.0 x 10⁵ Vero cells at an MOI of 0.1. At 24 hours post-infection, cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton-X100. VSV-EBOV-GP infected cells were immunostained with a polyclonal rabbit anti-VSV serum (Imanis Life Sciences) that recognizes internal VSV proteins and an Alexa Fluor® 488 secondary antibody. Plate scans were imaged on a Cellular Technology Limited ImmunoSpot analyzer and fluorescent focus units (ffu) were determined using a SpectraMax i3 plate reader.

**Growth of Filoviruses**

Filoviruses were grown on Vero E6 cells (ATCC, VA). Viral supernatants were harvested from tissue culture media and stored at -80 °C before use. Titers were determined by TCID50 (EBOV, RESTV, BDBV, SUDV) or by FFU (MARV) assays in Vero E6 cells. For TCID50, plates were monitored for CPE and fixed 5 to 7 days post infection. TCID50 values were calculated using the Reed-Muench method. For FFU assay, cells were infected for approximately 48 hours, fixed in 10% formalin, and prepared for antibody staining. The cells were permeabilized in 0.1% Triton X-100, washed, and blocked in 3.5% BSA. Infected cells were then stained with a polyclonal anti-MARV VLP antibody (IBT Bioservices 04-0005) and an AF488-conjugated goat anti-rabbit secondary antibody. Plates were imaged on a Cytation 1 Multimode Plate Reader.

**Filovirus neutralization assays**

Antibodies were diluted into culture medium containing 10% fetal bovine serum and incubated with virus for 1 hour at 37°C. Antibody concentrations ranged from 20 μg/ml to 0.5 pg/ml. The mixture was added to Vero E6 cells for 48 hours. Following incubation, cells were fixed in 10% neutral buffered formalin and virus was detected using smiFISH as previously described. Briefly, cells were permeabilized with 70% ethanol and fixed with 15% formamide. Infected cells were detected with oligonucleotides targeting viral mRNAs from each filovirus and a fluorescently labeled (Cy5) detection probe (Integrated DNA Technologies). Images were acquired on a Nikon Ti2 using a 10X lens in DAPI and Cy5 channels. Images were processed using a CellProfiler pipeline to quantify nuclear staining intensity and mRNA intensity for each image. Infection was quantified as viral mRNA intensity normalized to nuclear intensity for each well, and this was further normalized to the average of infection values from wells treated with the lowest dilution of antibody.

**Pseudoparticle generation**

EBOV GP pseudoparticles (EBOVpp) were generated as described. HEK293T cells were transfected with expression plasmids carrying the 2014 Zaire EBOV GP (KJ660346), lentivirus capsid proteins, and a lentivirus genome encoding a firefly luciferase under the control of the retrovirus long terminal repeat promoter. Supernatants were collected 48 hours post-transfection, clarified by centrifugation, and stored at -80 °C. Individual variants were cloned into 2014 Zaire EBOV GP (KJ660346) using site-directed mutagenesis and pseudotyped as described above.

**Pseudoparticle neutralization assays**

Pseudoparticle neutralization assays were performed by incubating EBOV pseudoparticles with serial dilutions of the indicated antibody. After 1 hour, the VLP/antibody mixtures were added to resuspended Huh7 (JCRB Cell Bank) and incubated for 72 hours.
Luciferase signal from the lentivirus reporter was detected using a BrightGlo luciferase assay kit (Promega) and a Victor X3 plate reader (PerkinElmer).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**NGS data analysis**
NGS analysis was performed using Array Studio software package platform (Omicsoft). Quality of paired-end RNA Illumina reads was assessed using the “raw data QC of RNA-Seq data suite”. Minimum and maximum read length, total nucleotide number, and GC% were calculated. Overall quality report was generated summarizing the quality of all reads in each sample, along each base pair. Paired-end RNA Illumina reads were then mapped against VSV-EBOV-GP virus genome using Omicsoft Sequence Aligner (OSA) version 4. Reads were trimmed by quality score using default parameters (when aligner encountered nucleotide in the read with a quality score of 2 or less, it trimmed the remainder of the read). OSA outputs were analyzed and annotated using Summarize Variant Data and Annotate Variant Data packages (Omicsoft). Target coverage was calculated for each sample. SNPs calling was performed using samples with average target coverage greater than 500 reads. SNPs with a minimum frequency of 1% and a coverage greater than 50 were identified and annotated.

**SPR analysis**
All specific SPR binding sensorgrams were double-reference subtracted as reported previously and the kinetic parameters were obtained by globally fitting the double-reference subtracted data to a 1:1 binding model with mass transport limitation using Biacore T200 Evaluation software v3.1 (Cytiva). The dissociation rate constant \( k_d \) was determined by fitting the change in the binding response during the dissociation phase and the association rate constant \( k_a \) was determined by globally fitting analyte binding at different concentrations. The equilibrium dissociation constant \( K_D \) was calculated from the ratio of \( k_d \) to \( k_a \). The dissociative half-life \( t_{1/2} \) in minutes was calculated as \( \ln(2)/(k_d \times 60) \).