Single-component multilayered self-assembling nanoparticles presenting rationally designed glycoprotein trimers as Ebola virus vaccines

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Ebola virus (EBOV) glycoprotein (GP) can be recognized by neutralizing antibodies (NAbs) and is the main target for vaccine design. Here, we first investigate the contribution of the stalk and heptad repeat 1-C (HR1C) regions to GP metastability. Specific stalk and HR1C modifications in a mucin-deleted form (GPΔmuc) increase trimer yield, whereas alterations of HR1C exert a more complex effect on thermostability. Crystal structures are determined to validate two rationally designed GPΔmuc trimers in their unliganded state. We then display a modified GPΔmuc trimer on reengineered protein nanoparticles that encapsulate a layer of locking domains (LD) and a cluster of helper T-cell epitopes. In mice and rabbits, GP trimers and nanoparticles elicit cross-ebolavirus NAbs, as well as non-NAbs that enhance pseudo-virus infection. Repertoire sequencing reveals quantitative profiles of vaccine-induced B-cell responses. This study demonstrates a promising vaccine strategy for filoviruses, such as EBOV, based on GP stabilization and nanoparticle display.

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Ebolavirus (EBOV), a member of the *Ebolavirus* genus in the *Filoviridae* family, can cause a severe human disease known as viral hemorrhagic fever. EBOV was solely responsible for the largest filovirus outbreak in history in 2013–2016 that caused 11,325 deaths. The EBOV outbreak in 2019 led to 2103 deaths and was declared an international emergency on July 17, 2019, by the World Health Organization (WHO). In recent years, significant progress has been made to counter this deadly virus. Neutralizing antibodies (NAbs) have provided effective therapeutic strategies for EBOV infection, as demonstrated by the ZMapp cocktail of murine chimeric antibodies, as well as human antibodies. Vaccines based on different delivery systems have been tested in humans14 as a promising vaccine platform. In contrast to the growing field was to display Env trimers on the cell surface26, is recognized by the humoral immune response and provides a promising vaccine platform for in vivo evaluation. To facilitate GP purification, we developed an immunoaffinity column based on mAb100, which is specific to native-like, trimeric GP. We first examined the contribution of two regions in GP, namely the stalk and heptad repeat 1-C (HR1C) regions, to GP metastability in a mucin-deleted Zaire EBOV GP construct (GPΔmuc). We extended the soluble GP ectodomain (GP^EC^) in the stalk region from residue 632 (C terminus of HR2) to 637 and introduced a W615L mutation based on a comparison of EBOV and MARV GPs. We also assessed eight proline mutations in HR1C, a region equivalent to the HR1 bend that is essential to HIV-1 Env metastability, for their ability to prevent the GP transition from pre- to postfusion states. Both the stalk and HR1C-proline mutations increased trimer yield, whereas the latter exhibited a complex effect on GP thermostability. In addition, newly engineered inter-protomer disulfide bonds (SS) were tested for their ability to increase trimer stability. Crystal structures were solved for two redesigned GPΔmuc trimers to validate the stalk and HR1C-proline mutations at the atomic level. We then displayed a redesigned GPΔmuc trimer on ferritin (FR), E2p, and I3-01 NPs. Locking domains (LDs) and helper T-cell epitopes were incorporated into E2p and I3-01 60-mers to stabilize the NP shell from the inside and create multilayered NP carriers. In mice and rabbits, GP trimer and NP vaccines induced distinct antibody responses with enhanced pseudovirus neutralization observed for some constructs. The next-generation sequencing (NGS) analysis of GP-specific B cells revealed different patterns for NPs that present large trimeric spikes versus smaller antigens, such as hepatitis C virus (HCV) E2 core. Our study thus reports on critical factors for EBOV GP metastability, single-component multilayered self-assembling protein NPs for the development of VLP-type vaccines, and a subunit vaccine strategy that is applicable to other filoviruses.

**Results**

**Tag-free immunoaffinity purification of EBOV GP trimers.** EBOV GP contains a heavily glycosylated mucin-like domain (MLD) that shields the glycan cap and neutralizing epitopes in GP1 and GP2 (Fig. 1a). A soluble, mucin-deleted form of Zaire EBOV GP (GPΔmuc) that produced the first high-resolution crystal structures was used as a basic construct to investigate GP metastability (Fig. 1a). In HIV-1 vaccine research, immunoaffinity chromatography (IAC) columns based on bNAb 2G12 and PGT145 have been widely used to purify native-like Env trimers. 2G12 targets a glycan patch on a single gp120, whereas PGT145 binds the trimer apex and can separate closed trimers from partially open and misfolded Env. These two bNAb columns have also been used to purify HIV-1 gp140-presenting NPs. Likewise, GP-specific IAC columns may provide useful tools for EBOV vaccine research. Corti et al. recently identified two potent NAb, mAb114 and mAb100, from a human survivor. Misasi et al. elucidated the structural basis for neutralization by mAb114, which targets the receptor-binding site (RBS), and mAb100, which interacts with the GP1/GP2 interface and internal fusion loop (IFL) of two GP protomers. Here, we examined the utility of mAb114 and mAb100 in IAC columns. The GPΔmuc constructs with and without a C-terminal foldon motif were transiently expressed in 250 ml HEK293F cells and purified on an antibody column prior to size-exclusion chromatography (SEC) using a Superdex 200 10/300 GL column and blue native polyacrylamide gel electrophoresis (BN-PAGE). With mAb114, both GPΔmuc samples showed aggregate (−9 ml), dimer (−12 ml), and monomer (−13.5–14 ml) peaks in the SEC profiles, but only GPΔmuc folded showed a visible trimer peak (−10.5–11 ml) in SEC and a band of slightly less than 440 kD on the BN gel (Fig. 1b). Following mAb100 purification, GPΔmuc produced a low overall yield, whereas GPΔmuc-folded demonstrated high trimer purity without any monomer or dimer peaks. Consistently, GPΔmuc-folded registered a single trimer band on the BN gel (Fig. 1c). Altogether, both mAb114 and mAb100 can
Fig. 1 Design and characterization of EBOV GPΔmuc trimers with modified HR2 stalk. a Left: Schematic representation of EBOV GP and GPΔmuc. GP1 N/C termini (GP1 N/C), mucin-like domain (MLD), internal fusion loop (IFL), heptad repeat regions 1/2 (HR1/HR2), transmembrane region (TM), and cytoplasmic tail (CT) are labeled with N-linked glycans, which are indicated as gray (mutated), red, and pink (within MLD) branches. Right: Ribbon representation of EBOV GP (PDB: 5JQ3) in transparent molecular surface, with GP1 in dodger blue and GP2 in yellow. The MLD and foldon are shown as a gray half oval and a green rectangle, respectively. b Schematic representation of mAb114 bound to EBOV GP (left), SEC profiles of mAb114-purified GPΔmuc and GPΔmuc-foldon (middle) from a Superdex 200 10/300 column, and BN-PAGE gel (right). c Schematic representation of mAb100 bound to EBOV GP (left), SEC profiles of mAb100-purified GPΔmuc and GPΔmuc-foldon (middle) from a Superdex 200 10/300 column, and BN-PAGE gel (right). In b, c, GP species (A aggregate, T trimer, D dimer, M monomer) are labeled on the SEC profile and BN-PAGE gel. d Ribbon representation of EBOV HR2 stalk (left) and MARV HR2 stalk (right) with CX6CC motif and residues of interest indicated. Middle: Logo analysis of EBOV and MARV HR2 sequences. e Schematic representation of GPΔmuc-W615L-L (or WL2)-foldon. f SEC profiles of 293F-expressed, mAb100-purified GPΔmuc-foldon and GPΔmuc-WL2-foldon from a HiLoad Superdex 200 16/600 column for three production runs. g Thermostability of GPΔmuc-foldon and GPΔmuc-WL2-foldon, with Tm, ΔT1/2, and Tn measured by DSC. h EC50 (μg/ml) values of EBOV GP-foldon, GPΔmuc-foldon, GPΔmuc-W615L-foldon, and GPΔmuc-WL2-foldon binding to 10 representative antibodies. Four pan-ebolavirus NAbs are colored in red. Antibody binding was measured by ELISA in duplicates, with mean value and standard deviation (SD) shown as black and red lines, respectively. Source data are provided as a Source Data file.
be used to purify EBOV GP, but mAb100 offers a more effective IAC method for purifying native-like trimers due to its recognition of a quaternary epitope. The stark difference in trimer yield between the two GPΔmuc constructs after mAb100 purification also suggests a strong tendency for trimer dissociation without the C-terminal foldon motif.

**Effect of HR2 stalk on EBOV GP metastability.** Previously, we demonstrated that the gp41 ectodomain (gp41ECTO) is the source of HIV-1 Env metastability. Unlike HIV-1 Env and EBOV GP, the gp41 HR2 helix is packed against the bottom of the gp41 HR1 helix and gp120 C1/C5 loops and forms extensive interactions at the gp140 trimer base. Even in the high-resolution GPΔmuc structures, the HR2 stalk still contains less helical content than most coiled-coils in the Protein Data Bank (PDB), ~15 versus ~30 aa, and becomes less regular and unwound toward the C terminus, suggesting an inherent instability in HR2. Most inward-facing amino acids were conserved except for W615 in EBOV and the equivalent L616 in MARV. Indeed, structural analysis revealed a critical difference at this position: the three HR2 stalk variants showed a W615L mutation may improve the stability of EBOV GP.

To further examine the effect of the HR2 stalk on GP trimer stability, we created three GPΔmuc constructs by replacing residues 617–632 with the coiled-coil from a GCN4 leucine zipper (Fig. 1a, left). Recently, King et al. solved a 3.17 Å-resolution structure for MARV GPΔmuc bound to a human mAb, MR191, with a ~9.0 Å distance of 10.5 Å and Cβ distance of 11.1 Å and Cβ distance of 9.0 Å; in contrast, with the smaller and more hydrophobic L616, a Cβ distance of 10.5 Å and Cβ distance of 8.3 Å were observed in MARV GP (PDB: 6BP2). This finding suggests that a W615L mutation may improve the stability of EBOV GP.

To determine the effect of the HR2 stalk on GP trimer stability, we created three GPΔmuc constructs by replacing residues 617–632 with the coiled-coil from a GCN4 leucine zipper (PDB: 2WPZ, residues 3–33) and by extending the C terminus to D637 and N643 to include a newly identified bNAb epitope that spans H2 and the membrane-proximal external region (MPER), termed “L” and “Ext”, respectively (Fig. S1a). Notably, D637 is also important for proteolytic cleavage by tumor necrosis factor α-converting enzyme, which enables GP to be shed from the virus surface. These three constructs were characterized by SEC and BN-PAGE following transient expression in 250-mL HEK293F cells and purification on an antibody column. With mAb114 purification, all three HR2 stalk variants showed a greater trimer yield than wildtype GPΔmuc in SEC (Fig. S1b), with trimer bands observed only for the stalk variants on the BN gel (Fig. S1c, left). Upon mAb100 purification, all three HR2 stalk variants showed more visible trimer bands than wildtype GPΔmuc on the BN gel (Fig. S1c, right). Of the three designs, “2WPZ” improved GP stability at the cost of replacing the entire HR2 stalk in GP2 but provided supporting evidence for the W615L mutation, which presumably increases coiled-coil content in the HR2 stalk (Fig. 1d). Overall, the “L” extension appeared to be a more practical solution as it modestly improved trimer stability with the inclusion of a recently identified bNAb epitope.

We next combined the W615L mutation and “L” extension in a single construct named GPΔmuc-W615L-I, or simply GPΔmuc-WL2-foldon (Fig. 1e). This construct, along with GPΔmuc-foldon, was transiently expressed in 1-L HEK293F cells and purified by an mAb100 column prior to SEC on a HiLoad Superdex 200 16/600 GL column (Fig. 1f). In three production runs, GPΔmuc-WL2-foldon consistently outperformed the wildtype construct, showing a twofold higher trimer peak in the SEC profile and a ~2.6-fold greater trimer yield after SEC (1.3 mg versus 0.5 mg). Thermostability was assessed by differential scanning calorimetry (DSC) for two purified GP trimers (Fig. 1g). The thermal denaturation midpoint (Tm) value for the stalk-stabilized trimer was 3 °C higher than the wildtype trimer (67 °C versus 64 °C). Stalk stabilization also increased the onset temperature (Ton) from 52.4 to 62.5 °C, with a narrower half width of the peak (∆T1/2) than the wildtype trimer (3.8 °C versus 5.1 °C). Antigenicity was assessed for four mAb100/SEC-purified EBOV GP trimers in the enzyme-linked immunosorbent assay (ELISA) (Fig. 1h and Fig. S1d, e). Ten antibodies were tested, including three NAbs targeting the base (KZ52, c2G4, and c1G7), two human NAb100 (IFL) and mAb114 (RBS), a non-NAb directed to the glycan cap (c13C6), and four pan-ebolavirus bNAb targeting the conserved HR2-MPER epitope (BDB223,74 and IFL1 – ADI-1594673,81 and CA4536,77). The GPΔmuc trimer showed notably improved antibody binding with respect to the GP41ECTO trimer, with an up to 7.6-fold improvement in the efficacy of human mAbs12,13,74–81 to 64 °C). Stalk stabilization also increased the onset temperature (Tm) from 52.4 to 62.5 °C, with a narrower half width of the peak (∆T1/2) than the wildtype trimer (3.8 °C versus 5.1 °C). Antigenicity was assessed for four mAb100/SEC-purified EBOV GP trimers in the enzyme-linked immunosorbent assay (ELISA) (Fig. 1h and Fig. S1d, e). Ten antibodies were tested, including three NAbs targeting the base (KZ52, c2G4, and c1G7), two human NAb100 (IFL) and mAb114 (RBS), a non-NAb directed to the glycan cap (c13C6), and four pan-ebolavirus bNAb targeting the conserved HR2-MPER epitope (BDB223,74 and IFL1 – ADI-1594673,81 and CA4536,77). The GPΔmuc trimer showed notably improved antibody binding with respect to the GP41ECTO trimer, with an up to 7.6-fold improvement in the efficacy of human mAbs12,13,74–81.
Fig. 2 Design and characterization of EBOV GPΔmuc trimers with modified HR1C bend. 

a Ribbon representation of EBOV GPΔmuc protomer (PDB: 5JQ3) in transparent molecular surface with GP1 in dodger blue, GP2 in yellow, and HR1C bends from three protomers in red. Left: side view. Right: top view.

b Left: Ribbon representation of the HR1 region in the prefusion (top, PDB ID: 5JQ3) and postfusion (bottom, PDB ID: 2EBO) states with the HR1 region in yellow and the HR1C bend in red. Right: Zoomed-in view of the HR1C bend with the eight residues in this region shown as sticks and labeled with the proline mutations, P1-P8.

c SEC profiles of mAb114-purified GPΔmuc-W615L-Pn variants (n=1–8) from a Superdex 200 10/300 column. SEC profiles of mAb100-purified GPΔmuc-W615L-P2 and -P4 are shown in the red dotted line. Trimer (T), dimer (D), and monomer (M) peaks are labeled on the SEC profiles for P2 and P4, with the trimer peak marked with a black dashed line.

d BN-PAGE gel of mAb114-purified GPΔmuc-WL2P2-foldon from a HiLoad Superdex 200 16/600 column (left) and BN-PAGE gel of SEC fractions 41–63 (55.5–62.0 ml) (right). SEC profiles were from three production runs.

eq  \begin{align*}
K_{D} & \text{ values of GPΔmuc-W615L-Pn variants (n=1–8)} \\
& \begin{array}{l}
\text{WT GPΔmuc-foldon} \\
\text{GPΔmuc-W615L-P2-foldon} \\
\text{GPΔmuc-W615L-P4-foldon} \\
\text{GPΔmuc-W615L-P6-foldon} \\
\text{GPΔmuc-W615L-P8-foldon} \\
\text{CA4S} \\
\text{BDBV223} \\
\text{AD1-15878} \\
\text{AD1-15946} \\
\text{KZG2} \\
\text{cG4} \\
\text{cG7} \\
\text{mAb100} \\
\text{mAb114} \\
\text{c13C6} \\
\text{\textsuperscript{a}} \\
\text{\textsuperscript{b}} \\
\end{array}
\end{align*}

\text{K}_{\text{D}} \text{ values from bi-layer interferometry (BLI) using six GP concentrations (400–12.5 nM). Octet sensorgrams are shown in supplementary Fig. S1h.}

f Thermostability of GPΔmuc-WL2P2-foldon and GPΔmuc-WL2P4-foldon, with \text{T}_{\text{m}}, \Delta \text{T}_{1/2}, \text{and } \text{T}_{\text{on}} \text{ measured by DSC.} 

h \text{KD values of GPΔmuc-foldon and GPΔmuc-WL2P2-foldon binding to 10 representative antibodies. BLI was performed on an Octet RED96 instrument using a trimer titration series of six concentrations (400–12.5 nM) by twofold dilution and kinetics (AHC) biosensors. The } \text{KD} \text{ values were calculated using a global fit 2:1 model. Source data are provided as a Source Data file.}
proline mutations in HR1\textsubscript{N} have effectively stabilized HIV-1 Env trimers\textsuperscript{60,68}. To examine this possibility, eight GP\textit{Δ}muc-W615L variants, each with a proline mutation in HR1\textsubscript{C} (termed P\textsuperscript{1} to P\textsuperscript{8}) but without the L extension and C-terminal foldon motif, were characterized by SEC following 250-ml 293 F expression and IAC. After mAb114 purification, most proline mutations showed little effect on the distribution of GP species except for T577P (P\textsuperscript{2}) and L579P (P\textsuperscript{3}), which appeared to have a trimmer peak at \texttildelow 11 ml in their SEC profiles (Fig. 2c). After mAb100 purification, only P\textsuperscript{2} and P\textsuperscript{4} produced any trimer yield, with a higher SEC peak observed for P\textsuperscript{2} that corresponded to well-formed trimers (Fig. 2c). The mAb100-purified GP was analyzed by BN-PAGE, which showed a trimmer band for P\textsuperscript{2} and P\textsuperscript{4} (Fig. 2d). Overall, the T577P mutation (P\textsuperscript{2}) can considerably increase trimer yield, whereas the L579P mutation (P\textsuperscript{3}) has a less pronounced effect.

Next, the T577P mutation (P\textsuperscript{3}) was incorporated into the GP\textit{Δ}muc-WL\textsuperscript{2}-foldon construct, resulting in a new design named GP\textit{Δ}muc-WL\textsuperscript{2}P\textsuperscript{3}-foldon. This construct was transiently expressed in 1-L 293 F cells and purified using an mAb100 column for SEC characterization on an HloLoad Superdex 200 16/600 GL column. In three production runs, GP\textit{Δ}muc-WL\textsuperscript{2}P\textsuperscript{3}-foldon generated a trimer peak that was twofold and fourfold higher than GP\textit{Δ}muc-WL\textsuperscript{2}-foldon and wildtype GP\textit{Δ}muc-foldon, respectively, with an average yield of 2.6 mg after SEC (Fig. 2e, f). Overall, the T577P mutation (P\textsuperscript{3}) can considerably increase trimer yield, whereas the L579P mutation (P\textsuperscript{3}) has a less pronounced effect.

Crystallographic analysis of redesigned EBOV GP\textit{Δ}muc trimers. To understand how the HR2 stalk and HR1\textsubscript{C} mutations affect EBOV GP, we determined crystal structures for the unliganded GP\textit{Δ}muc-foldon with WL\textsuperscript{2} and WL\textsuperscript{2}P\textsuperscript{2} at 2.3 and 3.2 Å, respectively (Fig. S3 and Fig. 3). Both proteins showed a three-lobed, chalice-like trimer structure\textsuperscript{40,66}. WL\textsuperscript{2}WL\textsuperscript{2}P\textsuperscript{2} yielded Ca root-mean-square deviations (RMSDs) of 0.9/1.6 Å (for 367/382 residues) at the single protomer level and 1.25/1.23 Å (for 1103/1145 residues) at the trimer level, respectively, relative to wildtype GP (PDB ID: 5JQ3)\textsuperscript{66}. WL\textsuperscript{2}WL\textsuperscript{2}P\textsuperscript{2} yielded a more complete structure than WL\textsuperscript{2} at the glycane cap (R302-V310) and HR2 stalk (I627-D637) (Fig. 3f and Fig. 3). In the WL\textsuperscript{2}P\textsuperscript{2} structure, the glycane cap covers the RBS with glycan moieties visible for N238/N257 in GP1 and N563 in GP2. In the WL\textsuperscript{2} structure, the glycane cap covers the RBS with glycan moieties visible for N238/N257/N268 in GP1 and N563 in GP2. The WL\textsuperscript{2} structure, the glycane cap covers the RBS with glycan moieties visible for N238/N257 in GP1 and N563/N618 in GP2 (Fig. 3b). GP1 consists mainly of β-strands, which form a broad semicircular groove that clamps the α3 helix and B19-B20 strands in GP2 (Fig. 3a). The T577P mutation appeared to have minimal effect on conformation of the HR1\textsubscript{C} bend, as indicated by a Ca RMSD of 0.2 Å for this 8-aa segment (Fig. 3b, left). In the WL\textsuperscript{2}P\textsuperscript{2} structure, the backbone carbonyl (CO) groups of R574, A575, and T576 in one protomer formed moderate-to-strong hydrogen bonds with the guanidinium moiety of R164 in an adjacent protomer, whereas only one CO–NH distance was within the 3.6 Å cutoff in wildtype GP\textit{Δ}muc\textsuperscript{66}. We previously hypothesized that a bulky, inward-facing W615 at the top of the coiled-coil destabilizes the HR2 stalk, whereas the W615L mutation would improve its packing (Fig. 1d). Indeed, W615L exerted a positive effect on the stalk structure (Fig. 3b, bottom center and right). The Ca-Ca/ Cβ-Cβ distances between two W615s of adjacent protomers in
wildtype GPΔmuc66, 11.1/9.0 Å, were reduced to 10.1/8.0 and 10.6/8.2 Å in WL2 and WL2P2, respectively (Fig. S3 and Fig. 3b, bottom center and right). As a result, the coiled-coil region in the EBOV HR2 stalk added one more helical turn (D624-V631), thereby resembling the MARV HR2 stalk (Fig. 1d, right). The L extension in the WL2P2 structure could be fully modeled to D637 as a well-ordered loop anchored to the C-terminal foldon motif (Fig. 3b, bottom center), rendering a complete HR2 stalk and
Fig. 3 Structural characterization of EBOV GP with stalk and HRL1c mutations. a The 3.2-Å-resolution crystal structure of EBOV GPΔmuc-WL2P2-foldon in a ribbon representation (top view and side view). GP1 is shown in dodger blue, except for the glycan cap, which is in gray. GP2 is shown in yellow with the internal fusion loop (IFL) in pink. N-linked glycans at N238, N257, and N563 are shown as sticks. b Ribbon representation of a GPΔmuc-WL2P2 protomer in the center with inset images showing structural comparison for the HRL1c bend (left), W615-surrounding HR2 region (bottom right), and C terminus of the HR2 stalk (right). For the HRL1c bend, WL2P2 is superimposed onto GPΔmuc (PDB ID: 5JQ3) (top) with three hydrogen bonds labeled for WL2P2 (middle) and GPΔmuc (bottom). For the W615-surrounding HR2 region, WL2P2 is superimposed onto GPΔmuc (PDB ID: 5JQ3) (top) with the coiled-coil structure shown for GPΔmuc (left) and WL2P2 (right). Ca and Cβ distances for residue 615 around the threefold axis are labeled. For the HR2 C terminus, WL2P2 is superimposed onto GP structures of SUDV (top), EBOV (middle), and BDBV (bottom) with Ca RMSDs calculated after fitting. The 2Fo–Fc electron density map contoured at 1σ is shown as a gray mesh for the WL2P2 protomer (center) and HR2 stalks (right).

Partial MPER. The superposition of HR2 stalks, from R596 up to D637, yielded Ca RMSDs of 1.5, 2.1, and 1.9 Å relative to EBOV-Mayinga (PDB ID: 5JQ3), Sudan ebolavirus (SUDV) (PDB ID: 3S88), and Bundibugyo ebolavirus (BDBV) (PDB ID: 6EA5) GPs, respectively (Fig. 3b, right), suggesting inherent structural variability in this region.

The WL2P2 structure was then compared to a recently reported Makona GPΔmuc structure (PDB ID: 6VKM, 3.5 Å) that contained the T577P/K588F mutation (Fig. S4). In total, 353 of 398 residues in the WL2P2 structure matched the double mutant with a Ca RMSD of 0.9 Å. A more complete cathepsin cleavage loop (between β13 and β14, residues 190–210) was observed in WL2P2 than in the double mutant, showing 10 more flanking residues, five on each side, of the loop that bridges over the IFL and interacts with IFL-directed NAbs such as mAb100. In addition, more electron density was observed for the β8 loop of the glycans cap (residues 294–310) in the stalk in WL2P2 than in the double mutant (Fig. S4b, top right). For the HRL1c bend, WL2P2 showed a Ca RMSD of 0.3 Å and more favorable hydrogen bonding patterns (Fig. S4b, bottom left). A Ca RMSD of 1.7 Å was obtained for the IFL region between the two structures (Fig. S4b, bottom right). Lastly, the WL2P2 structure was docked into a panel of known GP/antibody complexes (Fig. S5a). Overall, WL2P2 preserved all critical GP/antibody interactions (Fig. S5b). The GP/mAb100 complex is of most interest, because mAb100 was successfully used in this study to purify GP trimers. Cryo-EM revealed additional density near the mAb100 epitope both as a linker and as a built-in T-cell help in an HIV-1 Env T-cell epitope both as a linker and as a built-in T-cell help in an HIV-1 Env-13-01 NP construct, suggesting that additional structural and functional components can be incorporated into such large 60-meric NPs. Here, we sought to reengineer the E2p and I3-01 NPs by fusing a dimeric LD to the C terminus of an NP subunit and then a T-helper epitope to the C terminus of an LD (Fig. 4d). We hypothesized that each LD dimer can stabilize a non-covalent NP-forming interface from inside, and the T-cell epitopes can form a hydrophobic core at the center of a fully assembled NP. To test this hypothesis, we manually inspected 815 homodimers in the PDB and selected nine LDs of 100 residues or less (Fig. S6a). Based on structural compatibility, LDs 1–7 were tested for E2p, and five LDs (4–5 and 7–9) were tested for I3-01, all displaying GPΔmuc-WL2P2. Following transient expression in 100-ml ExpiCHO cells followed by mAb100 purification and SEC on a Superose 6 10/300 GL column (Fig. 4b). WL2P2 outperformed wildtype GPΔmuc with greater NP yield and purity. Based on molecular weight (m.w.), the SEC peaks at ~15 ml correspond to the unassembled GP-NP species, suggesting an inherent instability for wildtype E2p and I3-01. The mAb100-purified GPΔmuc-WL2P2-presenting NP samples were further analyzed by negative-stain EM (Fig. 4c), showing NPs mixed with impurities. Previously, we demonstrated the use of a pan-reactive T-cell epitope both as a linker and as a built-in T-cell help in an HIV-1 Env-I3-01 NP construct, suggesting that additional structural and functional components can be incorporated into such large 60-meric NPs. Here, we sought to reengineer the E2p and I3-01 NPs by fusing a dimeric LD to the C terminus of an NP subunit and then a T-helper epitope to the C terminus of an LD (Fig. 4d). We hypothesized that each LD dimer can stabilize a non-covalent NP-forming interface from inside, and the T-cell epitopes can form a hydrophobic core at the center of a fully assembled NP. To test this hypothesis, we manually inspected 815 homodimers in the PDB and selected nine LDs of 100 residues or less (Fig. S6a). Based on structural compatibility, LDs 1–7 were tested for E2p, and five LDs (4–5 and 7–9) were tested for I3-01, all displaying GPΔmuc-WL2P2. Following transient expression in 100-ml ExpiCHO cells and mAb100 purification, 12 LD-containing NP samples were characterized by SEC (Fig. 4e). Notably, LD4 and LD7 increased the NP peak (UV280 value) by 3 and 2.5-fold for E2p and I3-01, respectively, with substantially improved NP purity. The further incorporation of a T-cell epitope, PADRE, did not alter E2p properties but negatively impacted I3-01 (Fig. 4f). An I3-01 variant, termed I3-01v9 (or 1VLW-v9) in the previous study, was found to retain the NP yield and purity (Fig. 4f).
Seven GP-NP samples, with three variants for each 60-meric NP, were further analyzed by BN-PAGE (Fig. 4g). The FR and two E2p variants displayed a single high m.w. band corresponding to well-formed NPs, whereas the wildtype E2p and all three I3-01 samples showed additional low m.w. bands at 232–440 kD on the BN gel, indicating unassembled GP-NP species. Lastly, the mAb100/SEC-purified FR, E2p-LD4-PADRE (E2p-L4P), and I3-01v9-LD7-PADRE (I3-01v9-L7P) NPs that present the GPΔmuc-WL2P2 trimer were analyzed by negative-stain EM (Fig. 4h). In addition to well-formed particles in all three samples, an array of GPΔmuc spikes could be clearly recognized on the surface of FR and E2p-L4P NPs.

Antigenicity of the three GPΔmuc-WL2P2-presenting NPs was assessed by ELISA using the same panel of 10 antibodies (Fig. 4i).
and Fig. S6b, c). Compared with the WL2P2 trimmer, the three NPs exhibited an epitope-specific binding pattern. Overall, multivalent display improved antibody recognition of the RBS and glycan cap in GP1, but reduced binding for bNAbs that target the base and IFL at the GP1/GP2 interface (e.g., CA45) and the GP2 stalk (e.g., DDBV223). This finding raised concerns that some conserved bNAbs epitopes on the NP-displayed GP trimers may not be as accessible as on the soluble GP trimers. Two BLI experiments, with a total of three replicates used for the highest antigen concentration, were performed to further characterize the effect of multivalent display on the antibody recognition of various GP epitopes. Using comparable GP molar concentrations, the three NPs showed higher binding signals than the soluble trimers, with the most notable differences for NAbs mAb114 and mAb100 and a non-NAb c13C6. Based on these results, the FR, E2p-L4P, and I3-01v9-L7P NPs that present the redesigned GPmuc-WL2P2 trimer were selected for animal immunization.

Our results indicate that EBOV GP can be displayed on self-assembling NPs through gene fusion, which requires the optimization of both GP and NP. In addition to GPmuc, GP\textsubscript{ECTO} was also tested but found unsuitable for NP display, as confirmed by the EM analysis of a GP\textsubscript{ECTO}-10GS-FR construct (Fig. S6h). In this study, the multilayered NP design exploits the inner space of large, cage-like NPs to increase their stability and occlude antibody access to the base and stalk epitopes, which are targets of many bNAbs. This result may also be attributed to other factors, such as dosage, as noted in our recent study. The NP carrier accounts for 21–33% of the total mass of an NP vaccine, and the same antigen dose (50 μg) has been used for all groups except the I3-01v9-L7P NP group. Thus, mice in the NP groups would receive less GP antigen than mice in the trimer-only group.

Before analyzing the mouse plasma NAb response, we validated the pseudovirus (pp) neutralization assays by testing 10 antibodies against two ebolavirus strains, EBOV-Makona and DDBV-Uganda, in 293T and TZM-bl72 cells (Fig. 5c and Fig. S7d–f). mAb114 and early EBOV NAbs KZ25\textsuperscript{26} and c2G4, and c4G7\textsuperscript{10} only neutralized EBOV-Makona, whereas mAb100\textsuperscript{12} and four bNAbs, except DDBV223\textsuperscript{9}, blocked both ebolavirus-pp. ADI-15946 was the most potent bNAb, as indicated by the half maximal inhibitory concentration (IC\textsubscript{50}). Non-NAb c13C6, which is part of the ZMapp cocktail\textsuperscript{10} and binds the glycan cap\textsuperscript{45}, enhanced ebolavirus-pp infection of both cell types. When tested against pseudoviruses bearing the murine leukemia virus (MLV) Env, MLV-pps, all antibodies were non-reactive, except for c13C6, which enhanced MLV-pp infection in 293T cells (Fig. S7g). Overall, the enhanced observation for non-NAb c13C6 in the pseudovirus assays appeared to be consistent with ADE observed for human mAbs targeting the same epitope\textsuperscript{25}.

We next performed neutralization assays using purified mouse immunoglobulin G (IgG) from the last time point, w11 (Fig. 5d and Fig. S7h, i). Two distinct types of antibody response were observed: NAbs and non-NAbs that enhanced ebolavirus-pp infection. Among the three GP trimers, GP\textsubscript{ECTO} elicited a moderate NAb response with signs of enhancement noted for three mice, whereas an increase in both types of antibody response was observed for GP\textsubscript{muc}, suggesting that the removal of MLD exposes both NAb epitopes and the glycan cap, which is a main target for ADE-causing human mAbs\textsuperscript{25}. The stalk/HR1\textsubscript{C}
mutation WL2P2 appeared to have largely reversed the enhancement caused by MLD removal. Among the three NPs, E2p-L4P elicited primarily NAb responses that blocked both ebolavirus-pps, whereas enhanced pseudoviral infection was observed for one mouse in the FR group and for all mice in the I3-01v9-L7P group. Because only non-NAb c13C6 and not any of the (b)NAbs reacted with MLV-pps (Fig. S7g), here we utilized the MLV-pp assay to gauge glycan cap-directed non-NAb responses induced by different vaccine constructs (Fig. S7j). Indeed, the patterns of enhanced MLV-pp infection correlated nicely with the patterns of enhanced ebolavirus-pp infection (Fig. S7h–j). In the MLV-pp assay, E2p-L4P NP induced a minimum enhancement-causing
non-NAb response at a similar level to GP\textsubscript{ECTO} in which MLD shields the glycan cap and other GP epitopes from the humoral response. Our mouse study thus revealed some salient features of the GP-specific antibody response in the context of various GP forms and NP carriers. The c13C6-like non-NAbs that bind the glycan cap and cross-react with small secreted GP needed to be minimized in vaccine design. The high level of enhancement-causing non-NAb responses observed for GP\textsubscript{Muc} and I3-01v9-L7P may be explained by less trimeric GP and unassembled GP-NP species, respectively. Nonetheless, a multilayered E2p NP displaying 20 GP\textsubscript{Muc} trimers elicited a robust bNAb response in mice.

Immunogenicity of EBOV GP trimers and NPs in rabbits. Following a similar protocol, we assessed two GP\textsubscript{Muc} trimers, wildtype and WL\textsubscript{2P}, and three NPs presenting the WL\textsubscript{2P} trimer in rabbits (Fig. 5e). Rabbit sera collected at six timepoints during immunization were analyzed by ELISA using the same trimer probe (Fig. 5f and Fig. 58a, b). Notably, rabbits in the I3-01v9-L7P NP group were immunized with 20 μg of mAb100/SEC-purified material to reduce the enhancement-causing non-NAbs. Between the two trimer groups, WL\textsubscript{2P} showed higher average EC\textsubscript{50} titers for all time points except w11, with a modest P value of 0.0229 at w5. Among the three NP groups, the I3-01v9 and FR groups yielded the highest and lowest EC\textsubscript{50} titers, respectively, throughout immunization. A significant difference was found between the I3-01v9-L7P and E2p-L4P groups at w8, w11, and w13, with P values in the range of 0.0021–0.0053. Compared with the GP\textsubscript{Muc}-WL\textsubscript{2P} group, the I3-01v9-L7P NP group showed higher EC\textsubscript{50} titers at all six time points, with significant P values at w8, w11, and w13. In contrast, the FR and E2p-L4P groups yielded lower EC\textsubscript{50} titers than the GP\textsubscript{Muc}-WL\textsubscript{2P} group at w2 and w5, but this pattern was reversed at w8 and w11 with modest P values at w8. However, these two NP groups ended with lower EC\textsubscript{50} titers than the trimer group at the last time point, w13.

We then performed ebolavirus-pp and MLV-pp assays using purified rabbit IgG from w11 (Fig. 5g and Fig. 58c). At this time point, all vaccine groups showed NAb responses with no sign of enhanced pseudovirus infection, in contrast to the pattern of mixed antibody responses in mice (Fig. 5d). Notably, the I3-01v9-L7P NP group yielded higher average IC\textsubscript{50} titers than the other groups, 211.3 and 11.72 μg/ml for EBOV-Makona and BDBV-Uganda, respectively, supporting the notion that unassembled GP-NP species and not the I3-01v9 NP carrier were responsible for eliciting enhancement-causing non-NAbs in mice. All vaccine groups showed no sign of enhanced MLV-pp infection at w11 (Fig. 58c). Therefore, enhancement-causing non-NAbs appeared to be absent in rabbit sera toward the end of immunization. We next analyzed rabbit IgG from earlier time points at day 0 (Pre), w2, w5, and w8 (Fig. 58d–g), which revealed a unique temporal pattern of an increasing NAb response tailing a transient enhancement-causing non-NAb response. Specifically, enhanced MLV-pp infection was observed for the two trimer groups, FR group, and two multilayered NP groups at w2, w5, and w8, which then disappeared at w5, w8, and w11, respectively. Our longitudinal analysis suggests that vaccine-induced enhancement-causing non-NAbs may shift epitopes and gain neutralizing activity through gene conversion, a mechanism employed by the rabbit immune system to rapidly develop functional antibodies.

B-cell response profiles associated with EBOV GP trimers and NPs. Previously, we combined antigen-specific B-cell sorting and NGS to obtain a quantitative readout of the B-cell response induced by an HCV E2 core and its E2p NP. A more diverse usage of heavy-chain variable genes (V\textsubscript{H}), a higher degree of V\textsubscript{H} mutations, and a broader range of heavy-chain complementarity determining region 3 (HCDR3) length were observed for E2p65. In this study, we applied the same strategy to obtain GP-specific B-cell profiles (Fig. 6a). Using an Avi-tagged GP\textsubscript{Muc}-WL\textsubscript{2P}, 1TD0 probe (Fig. 59a), we sorted GP-specific splenic B cells from 25 mice (Fig. 59b), which were sequenced on Ion GeneStudio S5. The NGS data were analyzed using a mouse Antibodyomics pipeline (Fig. 59c), with quantitative B-cell profiles derived for different vaccine groups (Fig. 6b and Fig. 59d–f). We mainly focused on the GP\textsubscript{Muc}-WL\textsubscript{2P}-foldon group and multilayered E2p group to compare B-cell responses to GP\textsubscript{Muc} in the trimeric versus NP forms (Fig. 6b). In terms of germline gene usage, similar patterns were observed for V\textsubscript{H} and V\textsubscript{L} genes (Fig. 6b, panels 1 and 2). The redesigned GP\textsubscript{Muc} trimer activated more V\textsubscript{H}/V\textsubscript{L} genes (9.4/9.4) than its NP form (6.7), with P values of 0.0163 and 0.0076 for V\textsubscript{H} and V\textsubscript{L} genes, respectively. In contrast, the E2p NP decorated with 60 HCV E2 cores activated more V\textsubscript{H} but not V\textsubscript{L} genes than the E2 core65. In terms of somatic hypermutation (SHM), no significant difference was found between the two groups (Fig. 6b, panel 3). However, we observed a visible shift in the SHM distribution for the E2p-L4P NP group, which showed higher germline V\textsubscript{H}/V\textsubscript{K} divergence (on average 6.4%/2.9%) than the trimer group (on average 5.3%/2.6%). In the HCDR3 analysis, both average loop length and the RMS fluctuation (RMSF) of loop length were calculated (Fig. 6b, panel 4). Unlike in the HCV study, in which the RMSF of HCDR3 length yielded a P < 0.0001 between the E2 core and E2p NP groups, no significant difference was found between the EBOV GP\textsubscript{Muc} and E2p-L4P NP groups. Overall, EBOV and HCV NPs exhibited distinct patterns of the B-cell response with respect to their individual antigens. Notably, there were no apparent correlations...
between B-cell profiles and vaccine-induced NAb and enhancement-causing non-NAb responses. Our results thus suggest that antigen size, structure, glycosylation, and epitope distribution, other than the NP carrier, may contribute critically to the NP vaccine-induced B-cell response.

**Discussion**

With a mortality rate of up to 90%, EBOV has caused significant humanitarian crises, calling for action across many sectors to develop effective interventions. ZMapp established the use of NAbs as a treatment for patients with Ebola virus disease and propelled a community-wide campaign to identify NAbs and bNAbs. Several vaccine candidates have been tested in human trials. Of these, rSVV-ZEBOV demonstrated an efficacy of 100% in an open-label, cluster-randomized ring trial and was recently approved for human use. In a Phase 2 placebo-controlled trial of two vectored vaccines (including rSVV-ZEBOV), the antibody titer remained similar between the vaccine and placebo groups at 1 week post-vaccination and peaked after 1 month. A recent analysis of human B-cell responses to EBOV infection revealed hurdles in the GP-specific NAb response. NAbs from vaccinated humans showed low levels of SHM, suggesting a suboptimal B-cell response elicited by an otherwise effective vectored vaccine. The immune correlates of vaccine protection may not be universal and are likely determined by vaccine platforms, in addition to other factors. As most EBOV vaccines are based on viral vectors, protein subunit vaccines remain a promising approach to combat this deadly virus.

Here, we approached EBOV vaccine design with an antibody-based strategy focusing on GP optimization and multivalent display. We applied a similar strategy to develop HIV-1 and HCV vaccine candidates for in vitro and in vivo characterization, which provided a context for interpreting the findings for EBOV. Previously, we identified an HR1N bend as the cause of HIV-1 Env metastability and optimized an HCV E2 core. EBOV GP
belongs to the class-I fusion protein family\(^\text{33,34}\) and is inherently metastable, as is HIV-1 Env. In this study, we probed the cause of EBOV GP metastability by testing various designs that target the HR2 stalk, HR1\(_C\) bend, and GP/GP interface (via inter-protomer disulfide bonds). The detailed characterization revealed a stabilizing effect of the W615L mutation and stalk extension, in addition to the unexpected sensitivity of HR1\(_C\) (equivalent to HIV-1 HR1\(_A\)) to specific proline mutations, which increased the trimer yield but caused complex unfolding behaviors in DSC. Because this pattern was not reported for EBOV-Makona GPMuc that contained the T577P mutation\(^\text{34}\), GP metastability may thus be a strain-specific feature and warrant further investigation. Multivalent NP display proved to be challenging for EBOV GP because of its tendency toward dissociation. Although two-component NPs\(^\text{90,96}\) and the SpyTag/SpyCatcher system\(^\text{97,98}\) have been used to develop VLP-type vaccines, their inherent complexity in production, assembly, and purification, structural instability in vivo, and off-target response may dampen their potential as human vaccines. Here, we designed single-component, multivalved, and self-assembling protein NPs based on E2p and H5.019. Encapsulated within a single plasmid, such NP vaccines can be readily produced in bioreactor manufac-
tur(e) (GMP)-compatible CHO cells followed by IAC and SEC pur-
ification, providing a simple and robust manufacturing process. Our immunogenicity studies in mice and rabbits revealed some salient features of GP that need to be addressed in EBOV vaccine develop-
ment, regardless of the delivery platform. The choice of GPECTO or GPMuc as a vaccine antigen may lead to antibody responses that target different GP epitopes. Antibody access to GP epitopes at the IFL, base, and HR2 stalk may differ in the trimeric and NP forms. In animal immunization, we observed an ADE-like non-NAb response, which may be associated with non-trimeric GP and unassembled GP-NP species. Antibody isolation, structural epitope mapping, and live EBOV neutralization assays may be required to determine the biological relevance of these findings. Furthermore, EBOV challenge in rodents may help determine protective NAB titers elicited by GP-presenting NPs with respect to recombinant VLPs\(^\text{60-62}\). Nonetheless, the E2p-L4 NP elicited a minimum non-
NAB response in mice and the highly purified I3-019-L7 NP induced the strongest NAB response in rabbits, providing two promising constructs for further optimization and in vivo evaluation.

Having assessed various GP trimer and NP constructs, future investigation may be directed toward assessing other GPMuc designs, such as GPMuc-WL\(_7\) and GPMuc-SS2, as well as their NPs, to further improve NAB responses and reduce glycan cap-
directed non-NAB responses. The structural characterization of NABs and non-NABs isolated from immunized animals will provide critical insights into epitope recognition and guide future vaccine design. The strategy described in this study may find applications in vaccine development for other filoviruses.

**Methods**

**Design, expression, and purification of EBOV GPMuc and GPMuc-presenting NPs.** The glycopenin sequence of Zaire EBOV (Mayinga-76 strain) with a T42A substitution was used to design all GP constructs in this study (UniProt ID: Q05320), with the primers summarized in Table S1. Logo analysis of EBOV and MARV GP sequences was performed using WebLogo v2.8 software to facilitate the design of the W615L mutation. Structural modeling was performed using the UCSF Chimera v1.13 software to facilitate the design of HR1\(_C\)-proline and inter-protomer disulfide bond mutations. Wildtype and redesigned GPMuc constructs were transiently expressed in HEK293F cells (Thermo Fisher) for bio-
chemical, biophysical, and antigenic analyses. Briefly, HEK293F cells were thawed and incubated with FreeStyle\(^\text{TM}\) 293 Expression Medium (Life Technologies, CA) was mixed with 5 ml of PEI-MAX (1.0 mg/ml) in 25 ml of Opti-
MEM. After 30 min of incubation, the DNA-PEI-MAX complex was added to 1-L

293 F cells. Culture supernatants were harvested 5 days after transfection, clarified by centrifugation at 1126 \(x\) g for 22 min, and filtered using a 0.45 \(\mu\)m filter (Thermo Fisher). GPMuc proteins in the filtrate were extracted from the supernatant using mAb114 antibody column or mAb100 antibody column. Bound proteins were eluted three times, each with 5 ml of 0.2 M glycine (pH 2.2) and neutralized with 0.5 ml of Tris-Base (pH 9.0), and buffer-exchanged into phosphate-buffered saline (PBS, pH 7.2). Proteins were further purified by SEC on a Superdex 200 Increase 10/300 GL column (GE Healthcare). GPMuc-presenting NPs were produced in ExploCHO cells (Thermo Fisher).

Briefly, ExploCHO cells were thawed and incubated with ExploCHO\(^\text{TM}\) Expression Medium (Thermo Fisher) in a shaker incubator at 37 °C at 150 rpm with 8% CO\(_2\). When the cells reached a density of 10 \(\times\) 10\(^6\) ml\(^{-1}\), ExploCHO Expression Medium was added to reduce cell density to 6 \(\times\) 10\(^6\) ml\(^{-1}\) for transfection. The ExploFectamine\(^\text{TM}\) CHO/plasmid DNA complexes were prepared for 100-ml transfection in ExploCHO cells following the manufacturer’s instructions. For these NP constructs, 100 \(\mu\)g of plasmid and 320 \(\mu\)l of ExploFectamine\(^\text{TM}\) CHO reagent were mixed in 7.7 ml of cold OptiFKO\(^\text{TM}\) medium (Thermo Fisher). After the first on day 1, ExploCHO cells were cultured in a shaker incubator at 33 °C at 115 rpm with 8% CO\(_2\) according to the Max Titer protocol with an additional feed on day 5 (Thermo Fisher).

Culture supernatants were harvested 13–14 days after transfection, clarified by centrifugation at 3724 \(x\) g for 25 min, and filtered using a 0.45 \(\mu\)m filter (Thermo Fisher). The neutralized 100 \(\mu\)l antibody column or 100 \(\mu\)l mAb100 antibody column. Bound antibodies were then eluted using 0.5 ml of 0.2 M glycine (pH 2.2). The resulting readouts were measured on a plate reader (Perki-
on). Antibody (5 \(\mu\l) of plasmid and 25 ml of Opti-MEM transfection medium (Life Tech-
nologies, CA) was mixed with 5 ml of PEI-MAX (1.0 mg/ml) in 25 ml of Opti-
MEM. After 30 min of incubation, the DNA-PEI-MAX complex was added to 1-L
of shifts recorded for a sensor loaded with antibody but not incubated with antigen and for a sensor without antibody but incubated with antigen. The Octet data were processed using the Octet Manager Software that was linked to the Octet software. Experimental data were fitted with the binding equations describing a 1:1 interaction to achieve the optimal fitting and determine the K_d values. For GP-presenting NPs, two BLI experiments, one testing six antigen concentrations and the other testing the highest antigen concentration in duplicates, were performed. Binding signals (bound fraction) as a function of concentration (determined from three replicates) were used to quantify the effect of multivalent NP display on GP-antibody interactions. Notably, the GPmuc-WL<sup>2</sup>foldon trimer was also measured using AHQ biosensors to facilitate comparisons with three NPs that present the GPmuc-WL<sup>2</sup>foldon trimer multivalently.

**Differential scanning calorimetry.** Thermal melting curves of wildtype and redesigned GPmuc trimers were obtained with a MicroCal VP-Capillary calorimeter (Malvern). The purified GPmuc protein produced from 293 F cells was buffer exchanged into 1× PBS and concentrated to 27–50 μM before analysis by the instrument. Melting was probed at a scan rate of 90 °C·h⁻¹ from 25 to 110 °C. Data processing, including buffer correction, normalization, and baseline subtraction, was conducted using the standardized protocol from Origin 7.0 software.

**Protein production, crystallization, and data collection.** Two Zaire EBOV GPmuc-foldon constructs, one with the W615L mutation and Superdex 200<sup>16/600</sup> column (GE Healthcare). PBS filtration buffer during the purification process.

**Structure determination and refinement.** The structures of EBOV GP were determined by molecular replacement (MR) using Phaser<sup>103</sup> from the CCP4 suite<sup>104</sup> with the coordinates of Zaire Ebo Eb (PDB ID: 9QSO) and the model MOLREP<sup>105</sup>. The polypeptide chains were manually adjusted into electron density followed by SEC on a HiLoad Superdex 200 16/600 column (GE Healthcare). PBS filtration buffer during the purification process.

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5 min at 4 °C, followed by the addition of 2.5 μl of anti-mouse IgG fluorescently labeled with FITC (Jackson ImmunoResearch, catalog no. 115-095-071) and incubated for 15 min at 4 °C. Finally, 5 μl of premium-grade allyloxycyanin (APC)-labeled streptavidin was added to cells and incubated for 15 min at 4 °C. In each step, the cells were washed with PBS and the sorting buffer was 0.5 ml of FACS buffer. FITC+ APC+ GFP-specific mouse B cells were sorted using MoFloAria into one well of a 96-well plate with 20 μl of lysis buffer. Gating strategies used in antigen-specific mouse B-cell sorting are exemplified by the flow-chart in Fig. S9b. Briefly, antigen-specific mouse spleen B cells were isolated by gating on single cells that were live/dead marker negative, mouse IgG positive, and biotinylated EBOV GP positive. Flow cytometry data were collected using the Summit v6.3 software.

NGS and bioinformatics analysis of mouse B cells. Previously, a 5′-rapid amplification of cDNA ends (RACE)-polymerase chain reaction (PCR) protocol was reported for the unbiased sequencing of mouse B-cell repertoire65. In this study, this protocol was applied to analyze bulk-sorted, GP-specific mouse splenic B cells. Briefly, 5′-RACE cDNA was obtained from bulk-sorted splenic B cells of each mouse with the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (TaKaRa). The IgG PCRs were set up with Platinum Taq High-Fidelity DNA Polymerase (Life Technologies) in a total volume of 50 µl, with 5 µl of cDNA as the template, 1 µl of 5′-RACE primer, and 1 µl of 10 µM reverse primer. The 5′-RACE primer contained a PGM/SS P1 adaptor, while the reverse primer contained a PGM/SS primer. The SMART-Seq v4 cDNA library was amplified by PCR (25′C/33°C, inner primers V′ and mC outer primer as reverse primers for the 5′-RACE PCR processing of heavy and light (k) chains (Table S1). A total of 25 cycles of PCR was performed, and the expected PCR products (500–600 bp) were gel purified (Qiagen). NGS was performed on the Ion SS GeneStudio system. Briefly, heavy and light (k) chain libraries from the same mouse were quantitated using a Qubit® 2.0 Fluorometer with Qubit® dsDNA HS Assay Kit and then mixed at a 3:1 ratio before being pooled with antibody libraries of other mice at an equal ratio for sequencing. Template preparation and (Ion 530) chip loading were performed on Ion Chef using the Ion S5 GeneStudio system. Brie.

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**Competing interests**

The authors declare no competing interests.

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