Vaccine-Mediated Induction of an Ebolavirus Cross-Species Antibody Binding to Conserved Epitopes on the Glycoprotein Heptad Repeat 2/Membrane-Proximal External Junction

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The membrane-proximal external regions (MPER) of the human immunodeficiency virus envelope glycoprotein (GP) generate broadly reactive antibody responses and are the focus of vaccine development efforts. The conservation of amino acids within filovirus GP heptad repeat region (HR)2/MPER suggests that it may also represent a target for a pan-filovirus vaccine. We immunized a cynomolgus macaque against Ebola virus (EBOV) using a deoxyribonucleic acid/adenovirus 5 prime/boost strategy, sequenced memory B-cell receptors, and tested the antibodies for functional activity against EBOV GP. Antibody ma-C10 bound to GP with an affinity of 48 nM and was capable of inducing antibody-dependent cellular cytotoxicity. Three-dimensional reconstruction of single-particle, negative-stained, electron microscopy showed that ma-C10 bound to the HR2/MPER, and enzyme-linked immunosorbent assay reveals it binds to residues 621–631. More importantly, ma-C10 was found to bind to the GP of the 3 most clinically relevant Ebolavirus species, suggesting that a cross-species immunogen strategy targeting the residues in this region may be a feasible approach for producing a pan-filovirus vaccine.

Keywords. Ad5 immunization; cynomolgus macaques; Ebolavirus; MPER.

Ebola virus (EBOV) belongs to the filovirus family, which includes 5 Ebolavirus species (EBOV, Bundibugyo virus, Sudan virus, Reston virus, and Taï Forest virus) and one species of Marburgvirus [1]. Infection with EBOV causes severe hemorrhagic fever with fatality rates ranging from 50% to 90% [2]. The envelope glycoprotein (GP) of EBOV is a trimer of disulfide-linked heterodimers, GP1 and GP2. The GP1 subunit contains a large heavily glycosylated mucin-like domain and mediates engagement to the cellular viral receptor, NPC1. After receptor engagement by GP1, the GP2 subunit mediates fusion between the viral and endosomal cell membranes [3, 4]. The GP2 heptad repeat regions 1 and 2 (HR1 and HR2) are involved in complex structural rearrangements that result in exposure of an internal fusion peptide that mediates fusion of viral and host cell membranes. The HR1/HR2 and membrane-proximal external region (MPER) of the human immunodeficiency virus (HIV)-1 gp40 subunit are targets for fusion inhibitors [5] and

broadly cross-reactive antibodies [6–11]. The EBOV HR1/2 and MPER (Supplementary Figure 1A) have also been shown to participate in viral fusion [12] and are conserved across filoviruses. Therefore, these regions may represent a potential target for pan-filovirus vaccine development. Although several anti-HR2 monoclonal antibodies (mAbs) isolated from survivors of EBOV infections have been reported, there is as yet no evidence that vaccination can induce this class of antibodies [13–16]. In this report, we show that vaccination against EBOV GP is able to induce cross-species antibodies targeting the HR2/MPER junction by using binding, functional, and structural analyses.

METHODS

Ethics Statement

The study was approved by the Vaccine Research Center Institutional Animal Care and Use Committee.

Vaccination and Sample Preparation

One cynomolgus macaque (*Macaca fascicularis*) was primed with deoxyribonucleic acid (DNA) and vaccinated 3 additional times with recombinant adenovirus serotype 5 (rAd5) encoding for the EBOV GP as previously described [17, 18]. Peripheral blood mononuclear cells (PBMCs) were purified 1 month after the final boost, as described previously [19].

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Generation of Fluorescently Labeled Ebola Virus-Specific B-Cell Probes for Flow Cytometry

Transmembrane-deleted ($\Delta 657-676$) EBOV GP ($GP_{\Delta TM}$) and mucin-deleted GP ($\Delta 309-489$, $\Delta 657-676$) ($GP_{\Delta MUC}$) supplemented with a GCN4 site followed by an Avitag peptide (underlined) (MKQIEDKIEEIL SKIYHIENEIARIK KLIGEVASSS GLNDIFEAQKIEWHE AHHHHHHG) were produced by DNA synthesis and cloned into the pCAGGS expression vector (Integrated DNA Technologies). A transmembrane-deleted EBOV $GP_{\Delta TM}$ ($\Delta 657-676$) and EBOV $GP_{\Delta MUC}$ ($\Delta 309-505$, $\Delta 657-676$) were amplified by polymerase chain reaction (PCR) using 5'ATGGTACCTAAATGGGCGTTACAGGA and CGACGCGTTCCAATACCTGCCGGT and cloned into pCAGGS-GCN4-Avi. The EBOV Avitag proteins were expressed in HEK293T cells and purified as described previously [19, 20]. Purified proteins were biotinylated using the BirA enzyme kit (Avidity) following the manufacturer's instructions, and unbound or excess biotin was removed by using Zeba spin desalting columns (Thermo Scientific). Finally, biotinylated proteins were conjugated at a 1:1 molar ratio to ExtrAvidin-phycoerythrin (Sigma) and Streptavidin-APC (Life Technologies) for $GP_{\Delta TM}$ and $GP_{\Delta MUC}$, respectively.

Monoclonal Antibody Isolation From Antigen-Specific Single-Sorted Memory B Cells

Antigen-specific single memory B-cell sorts, amplification of VDJ/VJ genes by single-cell PCR, cloning into immunoglobulin (Ig) expression vectors, and mAb production were performed as previously described [21]. VDJ and VJ gene lineage assignments for each sequence were determined by alignment to known macaque V, D, and J genes using IMGIT/V-QUEST (<http://www.imgt.org>).

Production of Purified Proteins

Production of purified EBOV $GP_{\Delta TM}$, $GP_{\Delta MUC}$, thermolysin-cleaved GP (GP_{THL}), and secreted GP (sGP) was performed as previously described [19].

Enzyme-Linked Immunosorbent Assay

Gross binding epitope mapping and cross-species reactivity analysis for ma-C10 was performed by enzyme-linked immunosorbent assay (ELISA) using 100 ng/well of purified EBOV $GP_{\Delta TM}$, EBOV $GP_{\Delta MUC}$, EBOV GP_{THL} , EBOV sGP, Bundibugyo GP (IBT Bioservices), Reston GP (IBT Bioservices), Sudan GP (IBT Bioservices), and Marburg (Musoke) GP (IBT Bioservices). Gross epitope mapping controls for base-binding mAbs (eg, KZ52 and mAb100) and EBOV GP1 core binding (eg, mAb114) were included as positive controls in EBOV GP binding assays, and mAb114 was used as a positive control in EBOV sGP binding assays [20, 22]. The anti-HIV-1 Gp120 mAb VRC01 was used as a negative control in the gross epitope mapping and cross-reactivity ELISAs. The secondary antibody in both assays was goat antihuman IgG horseradish peroxidase (HRP)

Southern Biotech), and assays were developed as previously reported [22]. Epitope mapping was also performed by ELISA using 15-mer synthesized peptides with 5-mer overlap, covering the whole of EBOV GP2 (100 ng/well of each peptide and 800 ng/well of mAb) (Boston Bioscience). Binding of ma-C10 to EBOV $GP_{\Delta MUC}$ was included as a reference, whereas binding of mAb114 to LEIKKPDGSE was used as a positive peptide control [20]. In contrast, binding of ma-C10 to uncoated wells was used as a negative control for background binding in the peptide ELISA assay. The assays were performed in duplicate as previously described [23].

Western Blotting

Western blot binding of ma-C10 to EBOV GP was performed using 100 ng/well of purified EBOV $GP_{\Delta TM}$, $GP_{\Delta MUC}$, or GP_{THL} with a concentration of 1 μ g/mL of ma-C10. Polyclonal vaccinated macaque serum (1:2000 dilution) was used for the GP1 binding control blots. $GP_{\Delta TM}$ and $GP_{\Delta MUC}$ contain a C-terminal His tag. Anti-6 $\times$ -His tag mAb (HIS.H8; Thermo Fisher Scientific) was used for the GP2 control blots. GP_{THL} was not included as a GP2 control because we observed that GP cleavage with thermolysin removes the His tag. Cross-reactivity of ma-C10 was assessed for Bundibugyo, Reston, Sudan (Boniface) EBOV GP, and Marburgvirus (Musoke). The Bundibugyo, Reston, Sudan, and Marburg GP were purchased from IBT, whereas the Zaire and Sudan GP were made as previously described [24]. Rabbit polyclonal sera with known cross-reactivity with the GP2 subunit (IBT) were used as a control for detection of Sudan EBOV and Marburg virus GP. Due to the lack of available reagents to detect Bundibugyo and Reston GP, staining with Bio-Safe Coomassie G-250 Stain (Bio-Rad) is shown instead for these proteins. Secondary immunoblot staining was detected as previously described using a goat anti-human-HRP mAb (Southern Biotech) or a donkey anti-rabbit-HRP mAb (GE Healthcare) [19].

Electron Microscopy and Single-Particle Analysis

Samples were diluted as appropriate, adsorbed to glow-discharged carbon-coated copper grids, washed with several drops of buffer containing 20 mM HEPES, pH 7.0, and 150 mM KCl, and negatively stained with 0.75% uranyl formate, pH 5. Micrographs were recorded at a 0° and 45° (for $GP_{\Delta MUC}$ in complex with ma-C10 Fab only) tilt at a magnification of 100 000 on a FEI Tecnai T20 microscope operated at 160 or 200 kV and equipped with a 2k $\times$ 2k Eagle CCD camera. The pixel size was 2.22 Å. Serial electron microscopy (EM) [25] was used for data collection. For $GP_{\Delta MUC}$, 2-dimensional (2D) classification of 5319 particles was performed using Relion 1.4 [26]. For 2D and 3D analysis of the GP ΔMUC /ma-C10 Fab complex, the 45° tilted micrographs were divided into slices parallel to the tilt axis of the microscope for contrast transfer function correction. A total of 18 598 particles were picked with e2boxer from EMAN2.1 [27] using a box

size of 168×168 pixels. Reference-free 2D classification was performed using reference-free alignment and correspondence analysis in SPIDER [28]. The resulting 100 classes were inspected visually. Classes lacking sharp features or composed of noise were excluded, which reduced the number of particles in the dataset to 14785. The remaining classes were used to generate an initial model in EMAN2, which was then used as the reference volume for 3D reconstruction and refinement using reference projections in SPIDER [29]. The reconstruction and refinement were performed without symmetry and with imposed C3 symmetry. Imposing the symmetry resulted in a biologically incorrect 3D map with no continuous density between the base and the apex of the GP (data not shown). When no symmetry was imposed, the final map contained 1 GP trimer and 2 Fab fragments. The resolution of this map was 23 Å when a Fourier shell correlation (FSC) threshold of 0.5 was used. Reconstruction and refinement in EMAN2 with or without symmetry produced nearly identical results (data not shown). The refined model obtained with SPIDER was low-pass filtered and used, along with the corresponding particle stack, as input for the 3D refinement procedure of FREALIGN [30] with separation into three 3D classes [31]. The final resolution of the best-looking class (4784 particles) was 29 Å when an FSC threshold of 0.5 was used. In addition to 1 GP trimer and 2 Fab fragments, this map also displayed additional density compatible in terms of size and shape with a GCN4 trimerization domain. Forward projections of the map corresponded well to the 2D classes. UCSF Chimera [32] was used for map visualization and manual docking of molecular models.

Bio-Layer Interferometry to Assess Binding Kinetics at Neutral and Low pH

Binding kinetics of ma-C10 (Fabs) to GP_{ΔMUC} and GP_{THL} were determined based on bio-layer interferometry using a fortéBio Octet HTX instrument as previously described [20, 22].

In Vitro Neutralization and Antibody-Dependent Cell-Mediated Cytotoxicity Assay

ma-C10 was assessed for neutralization potency using a single-round infection assay with EBOV GP (Mayinga variant)-pseudotyped lentivirus particles as previously described [22]. The antibody-dependent cell-mediated cytotoxicity (ADCC) assay was conducted with a modified method as previously described [33, 34]. Briefly, rAd5 EBOV GP-transduced HEK293T cells were used as targets cells in the assay and labeled with carboxyfluorescein succinimidyl ester (CFSE) (Vybrant CFDA SE Cell Tracer Kit; Invitrogen). The CFSE-labeled EBOV GP-expressing cells were plated in duplicate wells in a V-bottomed 96-well plate at 5000 cells/well. Target cells were incubated with antibody at indicated concentrations for 20 minutes at room temperature. mAb114 and anti-HIV-1 VRC01 were used as positive and negative ADCC activity controls, respectively [22]. Effector cells resuspended in Roswell Park Memorial Institute medium were then added to the target cells at a 1:50 effector-to-target cell ratio. Each plate was incubated for 4 hours at 37°C, 5% CO₂. After 4 hours, plates were centrifuged at 250 ×g. Cells were stained with viability dye VIVID (in-house reagent) for 15 minutes, washed with phosphate-buffered saline + 2% fetal bovine serum, before fixation with 1% paraformaldehyde and analysis via flow cytometry. Thirty thousand nongated

Figure 1. ma-C10 enzyme-linked immunosorbent assay (ELISA) binding to transmembrane-deleted glycoprotein (GP_{ΔTM}), Fab binding kinetics, and antibody-dependent cell-mediated cytotoxicity (ADCC). (A) The ELISA data on binding of ma-C10 to purified GP_{ΔTM} is presented in red. The black curves are data from 2 known GP2/base binders, KZ52 and mAb100, and the GP1 binding antibody, mAb114. Open circles represent binding of the negative control, VRC01. (B) Bio-layer interferometry on the kinetics of binding for different concentrations of Fab from ma-C10 to mucin-deleted GP (GP_{ΔMUC}) at neutral pH. (C) Results of ADCC are indicated as percentage of killing (dead CFSE⁺ target cells incubated with the monoclonal antibodies [mAbs] and donor peripheral blood mononuclear cells). The black bar indicates ADCC for ma-C10, whereas gray bars indicate mAb114-positive and VRC01-negative controls at a concentration of 25.5 ng/mL. Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; EC50, half-maximal effective concentration; Fab, antigen-binding fragment.

events were acquired within 6 hours of the start of the ADCC assay using an LSRII cytometer (Becton Dickinson). After acquisition, analysis was performed using FlowJo software (TreeStar). The ADCC activity was determined by quantifying dead cells (VIVID⁺, CFSE⁺) out of the total CFSE⁺ population.

RESULTS

ma-C10 Binds to the Ebola Glycoprotein With Nanomolar Affinity and Mediates Antibody-Dependent Cellular Cytotoxicity

We vaccinated a cynomolgus macaque against EBOV GP using a DNA prime and Ad5 boosting strategy. B-cell receptors from memory B cells were sequenced and mAbs were produced. Antibodies were screened for binding against transmembrane-deleted GP (GP_{ΔTM}) using ELISA (Figure 1A, Supplementary Figure 1). One mAb, ma-C10 (Supplementary Figure 2), was found to bind to GP with a half-maximal

effective concentration (EC₅₀) of 1.6 ng/μL. We next tested affinity of ma-C10 for mucin-deleted GP (GP_{ΔMUC}). The K_D was noted to be 48 nM (Figure 1B), which is similar to previously published values for the ZMapp cocktail mAb 13C6 [20]. Similar to 13C6, ma-C10 did not show the capacity to neutralize infection in tissue culture (Supplementary Figure 3). Therefore, we asked whether ma-C10 was capable of mediating killing of infected cells by ADCC using a flow cytometry-based assay. Compared with the negative control antibody, we found that target-cell killing was substantially increased in the presence of ma-C10 (Figure 1C), indicating that ma-C10 is able to mediate ADCC.

ma-C10 Reacts With a Unique Epitope on the Ebola Virus Glycoprotein-2 Subunit

To map the ma-C10 binding region, we assessed binding by ELISA using purified protein variants of the EBOV GP:

Figure 2. ma-C10 enzyme-linked immunosorbent assay (ELISA) binding to mucin-deleted glycoprotein (GP_{ΔMUC}), thermolysin-cleaved GP (GP_{ΔTHL}), and secreted GP (sGP), Western blot, and bio-layer interferometry-based competition assay. (A) The ELISA data on binding of ma-C10 to purified GP_{ΔMUC} (top), GP_{ΔTHL} (middle), and sGP (bottom) are presented in red. The black curves are data from 2 known GP2/base binders, KZ52 and monoclonal antibody (mAb)100, and the GP1 binding antibody, mAb114. Open circles represent binding of the negative control, VRC01. (B) Western blot on ma-C10 was performed with purified transmembrane-deleted GP (GP_{ΔTM}), GP_{ΔMUC}, and GP_{ΔTHL}. ma-C10 shows reactivity with the GP2 subunit in all 3 constructs (top panel). Because both GP_{ΔTM} and GP_{ΔMUC} contain a 6×-His tag for purification placed on the GP2 subunit, an anti-6×-His tag mAb was used to control for GP2 binding. Binding of polyclonal serum is also shown as an example for binding to GP1 (bottom panels). (C) Bio-layer interferometry-based competitive binding assay to soluble GP_{ΔMUC} using the indicated mAbs. Biosensors were preloaded with GP_{ΔMUC} followed by the competitor and analyte mAbs as indicated. The percentage of inhibition is shown. ma-C10 did not compete with any of the other GP_{ΔMUC}-binding mAbs tested, including known base binders such as KZ52 and mAb100. Abbreviations: EC₅₀, half-maximal effective concentration; NB, not bound.

GP_{ΔMUC}, GP_{THL}, and sGP (Supplementary Figure 1). ma-C10 was able to bind all constructs except sGP (Figures 1A and 2A). Because the major difference between GP_{THL} and sGP is the lack of GP2, it indicated that ma-C10's binding site was likely contained in the GP2 subunit. To confirm binding of ma-C10 to GP2, we performed Western blot against GP_{ΔTM}, GP_{ΔMUC}, and GP_{THL}. The results confirmed that ma-C10 binds to the GP2 subunit (Figure 2B). We then assessed competition by bio-layer interferometry of ma-C10 with two GP2 base binders: KZ52, which binds 1 protomer at the interface between GP1 and GP2 [35], and mAb100, which binds the base of GP2 and makes contacts with 2 adjacent protomers [20]. The results showed that ma-C10 did not compete with either one of these mAbs for binding to GP_{ΔMUC} (Figure 2C), indicating that it has a distinct GP2-binding epitope.

ma-C10 Targets the Ebola Virus Glycoprotein Stalk

Because the probes used in the B-cell sorts included the entire GP ectodomain and there was a lack of competition with known GP2 base binders, we hypothesized that ma-C10 bound GP2 in a region below the base. To test this hypothesis, we performed negative-stain EM and single-particle analysis of trimeric GP_{ΔMUC} and ma-C10 Fab-trimeric GP_{ΔMUC} complex (Figure 3). In comparison with the 2D class averages of GP_{ΔMUC}, both the 2D class averages and a 3D map reveal that binding of ma-C10 to GP occurs below the base of GP in the GP2 stalk that contains

HR2 and MPER. Cryoelectron tomography previously showed the GP2 stalk as a narrow and elongated tail connecting GP to the viral membrane [36]. Therefore, this configuration may impose space constraints on the ability of antibodies to bind within this region. We noted that only 2 Fabs bound to trimeric GP_{ΔMUC} in all EM experiments (Figure 3). The fact that there were no appreciable amounts of GP trimers without any Fabs or only 1 Fab bound strongly indicates that binding was saturated, and the EM experiments revealed the actual binding stoichiometry. Furthermore, in the 3D map, the Fabs bind approximately 180° apart from each other (Figure 3C). This may be reflective of inherent structural constraints on epitope availability or reflect a distortion of the structure induced by the binding of the Fabs. Indeed, we note that there is an apparent asymmetry of the GP trimer in the EM map (Figure 3C). Although this may be due to a flattening of the complex caused by negative staining, it may also represent an asymmetry imposed on the trimer GP_{ΔMUC} by the binding of ma-C10 to the MPER region.

ma-C10 Binds to a Conserved Region of the Heptad Repeat-2/Membrane-Proximal External Junction of Ebola Virus Glycoprotein and Cross-React With Other Ebolavirus Species

The ability of ma-C10 to bind GP2 by Western blot indicates that the mAb targets a linear epitope on the EBOV GP2 subunit (Figure 2B). To determine the location of the epitope, we used ELISA to test for binding of ma-C10 to 15-mer

Figure 3. Negative-stain electron microscopy of mucin-deleted glycoprotein (GP_{ΔMUC}) in complex with ma-C10 Fab. (A) Two-dimensional (2D) class averages of the GP_{ΔMUC} trimer. Many classes contain an elongated extension below the base of the GP_{ΔMUC} trimer that is attributable to the heptad repeat region (HR)2, membrane-proximal external region (MPER), and GCN4 trimerization domain (white arrows). (B) The 2D class averages reveal 2 Fabs bound to 1 GP_{ΔMUC} trimer. Many classes contain additional elongated, flexible density extending below the binding site of the ma-C10 Fab that are attributable to the MPER and GCN4 trimerization domain. (C) A 3D reconstruction of GP_{ΔMUC} in complex with ma-C10. Crystal structures of Ebola GP (Protein Data Bank 5JQ3) [39] and the Fab fragment of Ebola virus antibody 114 (5FHA) [20] are modeled into the electron microscopy map. Two Fabs appear to be bound to the GP trimer in the GP2 stalk, with an approximately 180° angle between them. The top view suggests that the GP trimer in the complex deviates from the expected 3-fold symmetry. Abbreviation: Fab, antigen-binding fragment.

peptides, overlapping by 5, covering the whole of EBOV GP2. ma-C10 bound to 2 adjacent peptides with overlapping amino acids, DKIDQIIHDFV, and are located at the HR2/MPER junction (residues 621–631) (Figure 4A and B). In comparison to EBOV, this region is completely identical to Tai Forest and >80% identical to Bundibugyo (BDBV) and Sudan (SUDV). Percentage identity of EBOV residues at these positions is much lower in Reston and Marburg virus (Figure 4C). Therefore, we performed an ELISA binding assay using purified GPs from each virus (except Tai Forest) to evaluate for cross-reactivity. Consistent with the observed sequence homology in this region, ma-C10 showed high binding to BDBV, moderate binding to Reston (RESTV) and SUDV GP, and no binding to Marburg (MARV) (Figure 4D, Supplementary Figure 4A). As a further confirmation of

binding, we performed an immunoblot and found that ma-C10 reacted with denatured and reduced GPs from BDBV and SUDV but not RESTV and MARV, suggesting that the trimeric conformation of epitope is more important to RESTV than the other members of the *Ebolavirus* species (Supplementary Figure 4B).

DISCUSSION

For HIV-1, it has been demonstrated that the MPER is a region of vulnerability for cross-reactive antibodies across different HIV-1 quasiespecies [7–11]. This has resulted in the MPER being used as a potential target for both preventive vaccine development as well as for mAb-based therapeutics for ongoing HIV-1 infection. This may also be true for filoviruses due to the high degree of conservation within the GP2 subunit, including the

Figure 4. ma-C10 binds to a conserved region of the membrane-proximal external region (MPER) and cross-reacts with other *Ebolavirus* species. (A) Bar graph of enzyme-linked immunosorbent assay (ELISA) data showing specific binding of ma-C10 using 15-mer peptides with 5-mer overlap, shown for the last 80 amino acids of the GP2 subunit, which include the MPER and the transmembrane domain. The red bars indicate the positive hits for ma-C10. Binding of ma-C10 to mucin-deleted glycoprotein (GP Δ_{MUC}) and binding of mAb114 to LEIKKPDGSE [20] are included as positive controls. Binding shown as absorbance at 450 nm. (B) The peptide sequence overlap is shown for residues 613–671 of the GP2 subunit. The sequence DKIDQIIHDFV corresponding to the ma-C10 binding site has been highlighted in red. (C) Amino acid sequence alignment of residues 614–657 in the Ebola, Bundibugyo, Reston, and Sudan virus GP2 and Marburg virus GP2. The box indicates the binding site for ma-C10. Underlined in red are the amino acids different from the Zaire species. (D) Half-maximal binding (EC₅₀) values of ma-C10 calculated from ELISA binding assays to the GPs from Ebola (EBOV), Marburg (MARV), Bundibugyo (BDBV), Reston (RESTV), and Sudan (SUDV) viruses. Shown as compared with is the anti-human immunodeficiency virus gp120 antibody VRC01. The EC₅₀ values were calculated using a least squares fit 4-parameter nonlinear regression (Prism version 7). The values shown represent the average of 2 independent experiments (full data curves are shown in Supplementary Figure 4A).

region of the HR2/MPER junction identified in this study (ie, residues 621–631). Several antibodies targeting the HR of GP2 have previously been identified in 1 survivor of BDBV infection and 1 survivor of EBOV infection [13, 37, 38]. ADI-16061, isolated from an EBOV disease survivor, is thought to bind near the HR2/MPER junction [13, 37, 38]. ADI-16061 binds 3 Fabs per trimer and is capable of neutralizing EBOV and BDBV viruses but not SUDV. In contrast, ma-C10 binds at least 4 of the *Ebolavirus* species and all of the clinically relevant species that cause human disease (ie, EBOV, BDBV, and SUDV). Furthermore, an ADCC assay using cells bearing EBOV GP suggest that ma-C10 is capable of mediating EBOV GP-specific cell killing. In addition, the 2D and 3D EM analysis of ma-C10 binding indicates that ma-C10 Fab binding saturates at 2 Fabs per GP trimer, 180° apart, and the 3D map suggests the possibility that Fab binding may induce conformational changes to GP. Taken together, these data suggest that antibodies that target the HR2/GP2 regions may be able to act through both neutralization and/or effector mechanisms. The isolation of ma-C10 shows that both infection and vaccination are able to generate antibody responses to the HR2/MPER junction. To optimize vaccine designs targeting this region, it will be important to understand the underlying differences in binding to this region.

The EBOV's receptor-ligand interaction occurs in endosomes and is substantially different from viruses such as HIV-1, for which the receptor-ligand interaction occurs on the cell surface. The HR2 has been recently solved by x-ray crystallography [39], but little information is known about the MPER and the HR2/MPER junction due to their absence from published trimeric GP crystal structures [35]. Cryoelectron tomography data suggest that there would be limited access to the GP2 stalk [36]. Our observation that fewer than 3 Fabs interact with this region supports the hypothesis that there may be spatial constraints on binding. The presence of the viral membrane in *in vivo* infection may further restrain binding by full Igs.

The affinity of ma-C10 Fab for GP_{ΔMUC} is similar to that of other nonneutralizing mAbs in protective cocktails [20]. It is interesting to note that of 11 residues in the HCDR3, ma-C10 contains a WSW motif centrally and an additional tryptophan at the C-terminus of the HCDR3 (Supplementary Figure 2). Tryptophans have been shown to make critical stabilizing contacts with the viral membrane for the HIV-1 MPER-directed mAbs 10E8 and 4E10 [40, 41]. Thus, the finding of a tryptophan-rich region in ma-C10 warrants further evaluation to determine the potential for contacts between tryptophans in the HCDR3 and the viral envelope. If this lipid interaction with these domains is confirmed, it suggests that these motifs may be a general property of MPER-directed antibodies.

ma-C10 may participate in ADCC and other Fc-mediated mechanisms of virus *in vivo* that are independent of an *in vitro* neutralization phenotype. Indeed, nonneutralizing antibodies have been shown to be protective against EBOV disease in animal

models [42, 43]. Similarly, the requirement of CD8⁺ T cells for surviving viral challenge in vaccinated macaques [44] indicates that the killing of infected cells, rather than sterilizing immunity, may play an important role in controlling EBOV infection.

CONCLUSIONS

Given the conservation at the HR2/MPER junction and reactivity of ADI-16061 and ma-C10 against residues 621–632, this study suggests at least 1 site of vulnerability in GP2 that can be targeted by vaccines and suggests that HR2/MPER-directed vaccination may be a feasible strategy for producing a pan-filovirus immunization.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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