Pan-ebolavirus and Pan-filovirus Mouse Monoclonal Antibodies: Protection against Ebola and Sudan Viruses

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ABSTRACT

The unprecedented 2014-2015 Ebola virus disease (EVD) outbreak in West Africa has highlighted the need for effective therapeutics against filoviruses. Monoclonal antibody (MAb) cocktails have shown great potential as EVD therapeutics; however, the existing protective MAbs are virus species specific. Here we report the development of pan-ebolavirus and pan-filovirus antibodies generated by repeated immunization of mice with filovirus glycoproteins engineered to drive the B cell responses toward conserved epitopes. Multiple pan-ebolavirus antibodies were identified that react to the Ebola, Sudan, Bundibugyo, and Reston viruses. A pan-filovirus antibody that was reactive to the receptor binding regions of all filovirus glycoproteins was also identified. Significant postexposure efficacy of several MABs, including a novel antibody cocktail, was demonstrated. For the first time, we report cross-neutralization and in vivo protection against two highly divergent filovirus species, i.e., Ebola virus and Sudan virus, with a single antibody. Competition studies indicate that this antibody targets a previously unrecognized conserved neutralizing epitope that involves the glycan cap. Mechanistic studies indicated that, besides neutralization, innate immune cell effector functions may play a role in the antiviral activity of the antibodies. Our findings further suggest critical novel epitopes that can be utilized to design effective cocktails for broad protection against multiple filovirus species.

IMPORTANCE

Filoviruses represent a major public health threat in Africa and an emerging global concern. Largely driven by the U.S. biodefense funding programs and reinforced by the 2014 outbreaks, current immunotherapeutics are primarily focused on a single filovirus species called Ebola virus (EBOV) (formerly Zaire Ebola virus). However, other filoviruses including Sudan, Bundibugyo, and Marburg viruses have caused human outbreaks with mortality rates as high as 90%. Thus, cross-protective immunotherapeutics are urgently needed. Here, we describe monoclonal antibodies with cross-reactivity to several filoviruses, including the first report of a cross-neutralizing antibody that exhibits protection against Ebola virus and Sudan virus in mice. Our results further describe a novel combination of antibodies with enhanced protective efficacy. These results form a basis for further development of effective immunotherapeutics against filoviruses for human use. Understanding the cross-protective epitopes are also important for rational design of pan-ebolavirus and pan-filovirus vaccines.

The Filoviridae family consists of a single marburgvirus species with Marburg virus (MARV) and Ravn virus (RAVV), as well as five ebolavirus species, Ebola virus (EBOV), Sudan virus (SUDV), Bundibugyo virus (BDBV), Reston virus (RESTV), and Tai Forest virus (TAFV) (1, 2). Filoviruses cause lethal hemorrhagic fever in humans and nonhuman primates (NHPs), with case fatality rates of up to 90% (3, 4). EBOV has caused the majority of filovirus hemorrhagic fever outbreaks, including the 2014 outbreak in West Africa with more than 27,000 cases and 11,000 deaths (5). However, other members of Filoviridae have also caused human epidemics, including seven outbreaks of SUDV (6), two outbreaks of BDBV (6), and 12 outbreaks of MARV (7). RESTV has not caused disease in humans, but its recent detection in pigs has raised concern about the potential emergence of ebolaviruses in the human food chain (8). Thus, there is urgent need for the development of broadly protective filovirus therapeutics, as the nature of future outbreaks cannot be predicted.

Recent reports indicate that monoclonal antibodies (MAbs) against the filovirus glycoproteins (GP) represent effective postexposure treatments for Marburg virus and Ebola virus hemorrhagic fever (9–17). However, nearly all efficacious ebolavirus an-
tibodies are species specific, and the majority of them target EBOV. The primary amino acid sequence of GP shows nearly 30% identity between EBOV and MARV and 56 to 65% identity between the various ebolavirus species. Despite this homology, no cross-reactive antibodies have been described so far that would show cross-neutralization or cross-protective efficacy against multiple filovirus species.

Over the past few years, major progress has been made toward development of effective immunotherapeutics against EBOV. These studies indicated that antibodies targeting certain key epitopes within EBOV GP act synergistically to enhance the therapeutic effects. Two antibody cocktails, MB-003 (11) and ZMab (18), were first reported to show significant postexposure efficacy in NHPs. Upon systematic evaluation of various cocktails, two components of ZMab (4G7 and 2G4) and one component of MB-003 (13C6) were combined into a novel cocktail, referred to as ZMapp, which demonstrated 100% efficacy when administered as late as 5 days postinfection in NHPs (14). Recent studies using single-molecule electron microscopy revealed that 13C6 binds to a region on the top of the GP trimer known as the glycan cap, while 4G7 and 2G4 target a conformational epitope consisting of the GP2 subunit and the N terminus of the GP1 subunit within the base of the GP trimer (19) that overlaps with the site targeted by KZ52, a potent neutralizing antibody identified from an Ebolavirus disease (EVD) survivor (20, 21). This antibody cocktail identifies two epitopes as key sites of vulnerability on EBOV (19). However, it is not clear whether cross-reactive antibodies targeting these two sites can be developed. In fact, another MAb (16F6) targeting the same region within SUDV GP has been identified (22), but both 16F6 and KZ52 are strictly species specific. Thus, effective pan-ebolavirus or pan-filovirus antibodies, if possible, are likely to target novel epitopes.

Here we report a set of four mouse MAbs reactive to novel conformational epitopes conserved across different species of ebolavirus as well as a MAb broadly reactive to all filoviruses, including MARV. The pan-ebolavirus MAbs showed significant protective efficacy against EBOV in a mouse infection model. One of the antibodies potently neutralized both EBOV and SUDV and showed protection against both viruses in mice, the first demonstration of a single antibody providing in vivo protection against two ebolavirus species. Combination of two of these antibodies targeting a new epitope near or on the glycan cap and another potentially involving GP2 showed enhanced efficacy in mice, suggesting that effective pan-ebolavirus therapeutic antibody cocktails can be developed. Our studies further suggest that, besides neutralization, Fc-mediated effector functions play a role in protective efficacy of the antibodies.

MATERIALS AND METHODS

Production and purification of filovirus glycoproteins. Plasmids for the following glycoprotein (GP) constructs were generated: GPΔTM (GP ectodomain lacking the transmembrane region) for EBOV (Mayinga) and SUDV (Boniface) containing amino acids 1 to 627 with a hemagglutinin (HA) tag (YPYDVPDYA) followed by a factor 10a cleavage site (IEGRG). EBOV GPΔmuc (amino acids 1 to 311 followed by an aspartic acid and linked to residues 464 to 637); and SUDV GPΔmuc (residues 1 to 313 linked to residues 474 to 640) were generated and expressed in 293T cells. GPΔTM proteins were purified from the supernatants as described previously (20). Briefly, proteins expressed in 293T cells were purified using Q-Sepharose Fast Flow resin. Eluates were pooled and passed over a GE S-200 HR column. S-200 fractions containing GPΔTM were pooled and dialyzed against phosphate-buffered saline (PBS). GPΔmuc protein was purified using Q-Sepharose Fast Flow resin, and eluted fractions containing the proteins were pooled and dialyzed against PBS. For insect cell expression of GPΔTM for EBOV, SUDV, RESTV, and BDBV, the coding regions for amino acids 1 to 605 (with the exception that for BDBV, residues 1 to 609 were used) followed by a C-terminal 6×His tag were cloned into the baculovirus transfer vector pFastBac (Invitrogen). Bacmid DNA was produced as described previously (23) and used to transfect S9 insect cells to generate recombinant baculoviruses containing GPΔTM, which was further amplified in S9 cells. The final virus was used to infect S9 cells for purification of the proteins from the supernatants 3 days postinfection. After separation of cell debris, the supernatants were concentrated 10 times, mixed with 2 mM CaCl2, 0.25 mM Ni2+, 20% glycerol, 10 mM imidazole, 0.5% Triton X-100, and 1 M NaCl, and the pH was adjusted to 7.2. Ni beads (GE Healthcare Life Sciences) were added at 1 ml per liter of concentrated supernatant and mixed overnight at 4°C. The beads were separated by centrifugation, resuspended in PBS plus 0.2% Tween 20, and packed into a Bio-Rad Econo-column. The column was washed with the following buffers: PBS (pH 7.1) supplemented with 20% glycerol and 0.2% Tween 20, followed by PBS supplemented with 20% glycerol, 0.2% Tween 20, and 10 mM imidazole. Protein was eluted with 500 mM imidazole in PBS. Eluted proteins were dialyzed against PBS supplemented with 10% glycerol containing arginine and glutamic acid (pH 7.4). For production of soluble GP (sGP), the full coding sequence of EBOV sGP including delta peptide followed by a C-terminal tag was expressed in 293T cells. The supernatants were passed through a Ni column to separate the delta peptide. The flowthrough was concentrated and used as a source of sGP. Proteolytically cleaved GP ectodomains were produced in S2 cells as described previously (24).

Immunization of mice and generation of monoclonal antibodies. Female BALB/c mice (6 to 8 weeks old) were immunized with a combination of GPΔmuc proteins for EBOV (Mayinga), SUDV (Boniface), and MARV (Musoke) (25 μg each) on study days 0, 14, and 28 (group 1) or study days 0, 14, 28, 42, and 56 along with 20 μg Sigma adjuvant system by intramuscular (i.m.) route. An intravenous (i.v.) dose of the same antigens without adjuvant was administered on day 35 (group 1) or day 63 (group 2). Mice were euthanized on day 42 (group 1) or 64 (group 2), and their spleens and lymph nodes were harvested. Lymphocytes (1 × 108), which had been collected from spleens and lymph nodes, were fused with the 2 × 105 PEG2/0-Ag14 myeloma cells (ATCC), using polyethylene glycol (PEG) according to method A in the ClonaCell-HY hybridoma cloning manual (Stemcell Technologies) (25). Fused cells were incubated overnight in medium C to allow the fused cells to go through one cell cycle to express the hypoxanthine-guanine phosphoribosyltransferase (HRPT) enzyme that will allow them to survive in selection medium D. The day after the fusion, cells were plated in methylcellulose-containing medium D in 96-well plates and left undisturbed for 10 to 14 days at 37°C and 5% CO2. The cells on the plates were fed with medium E every few days. Supernatants were screened for antibodies reactive to EBOV, SUDV, and MARV glycoproteins. Cells from wells identified as positive against two or all three glycoproteins in the screening enzyme-linked immunosorbent assay (ELISA), were expanded and reconstituted by limiting dilutions using medium E.

Animal immunization studies and all associated experimental procedures were preapproved and performed according to guidelines set by the Noble Life Sciences (Gaithersburg, MD) Animal Care and USE Committee (protocol 12-05-017-IBT). This animal facility used is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and adheres to the principles stated in the Guide for the Care and Use of Laboratory Animals (26). Animals were euthanized in accordance with the 2013 American Veterinary Medical Association (AVMA) Guidelines on Euthanasia using carbon dioxide exposure, followed by cervical dislocation. The choice of numbers of mice per group (n = 3) is based on the minimum number of animals that is required to yield a consistent anti-
body response, generation of sufficient B cells for analysis, and generation of sufficient serum volumes for analysis.

Production and purification of MAbs. Hybridoma cells were grown in bioreactor flasks (CELLline CL350 [catalog no. 90010; Integra]). Supernatants were collected every 3 to 5 days and combined for purification using an Akta fast protein liquid chromatograph (FPLC). Produced antibodies were captured by protein A (catalog no. 17-0403-01; GE Healthcare) and washed and eluted with 0.1 M glycine buffer (pH 2.4). Fractions, containing the Ab peak were collected, neutralized with Ab buffer (20 mM L-histidine [pH 6.0], 150 mM NaCl, and 4% sucrose) and dialyzed against the same buffer overnight at 40°C.

ELISA. Purified glycoproteins were immobilized overnight at 100 ng/ well in PBS at 4°C on 96-well Nunc MaxiSorp plates (Thermo Fisher Scientific) and incubated with hybridoma supernatants or serial dilutions of purified MAb. Bound antibodies were detected using a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody and 3',5'-tetramethylbenzidine (TMB) substrate. Absorbance values determined at 450 nm were transformed using Softmax 4 parameter curve-fit (Molecular Devices). The half-maximal effective concentration (EC_{50}) value at the inflection point of the curve is reported.

Competition ELISAs. His-tagged EBOV GP ATM was immobilized at 100 ng/well on 96-well nickel-coated plates (Promega) at 4°C overnight. The next day, the plates were washed and blocked for 1 h, and a set of two MAb was then added and allowed to bind for 1 h at room temperature. To detect competition between mouse and humanized MAb, 1 μg/ml mouse MAb 4/254 (4 μg/ml for mBC4) was mixed with a 10-fold excess of humanized MAb and detected using goat anti-mouse conjugated to HRP. To detect competition between two mouse MAb, mBC4 was biotinylated with EZ-Link NHS-biotin according to the manufacturer's protocol (Life Technologies, Carlsbad, CA) and detected with antistreptavidin antibody conjugated to HRP. KZ52 competition with humanized MAb also required biotinylation and detection with anti-streptavidin antibody conjugated to HRP. After 1 h, TMB substrate was added, and absorbance values were determined at 650 nm on a VersaMax plate reader. Percent competition was calculated from MAb binding in the presence of an irrelevant MAb control. For mapping of linear epitopes, the competition assay was set up as described above and performed in the presence or absence of 100-fold molar excess of a set of overlapping peptides spanning the sequences of filovirus GP.

Neutralization assays with VSV-GP_{SUDV} and VSV-GP_{EBOV}. Neutralization assays were performed using vesicular stomatitis virus pseudotyped to display the GP from either SUDV or EBOV in place of its native G glycoprotein (VSV-GP_{SUDV} or VSV-GP_{EBOV}, respectively). The viral genome encodes an enhanced green fluorescent protein (eGFP). Infection is scored by counting fluorescent cells. The protocol for VSV-GP production has been described elsewhere (27).

Neutralization of live EBOV and SUDV. Antibodies were diluted in Vero growth medium (Earle's saline and L-glutamine, 5% fetal bovine serum [FBS], 1% penicillin-streptomycin) at two times the desired final concentrations, mixed with equal volume of live virus (EBOV or SUDV), and incubated at 37°C for 1 h before adding to Vero cells in 96-well black plates. The cells were incubated with MAb/virus inoculum (at a multiplicity of infection [MOI] of ~0.5) for 1 h at 37°C and washed with PBS, and growth medium alone without antibody was added to all wells. The cells were fixed at 48 h postinfection, and infected cells were determined by an indirect immunofluorescence assay (IFA) using virus-specific MAb (EBOV-specific KZ22, SUDV-specific 17F6) and fluorescently labeled secondary antibodies (goat anti-human IgG conjugated to Alexa Quor Flou 488; Molecular Probes). The percentages of infected cells were determined using an Operetta and Harmony software. Data are expressed as the percentage of inhibition relative to control cells treated with vehicle for both EBOV and SUDV.

Antibody-mediated cellular phagocytosis. (i) Cells. Murine monocytes (RAW cells) and human monocytes (THP-1 cells) were cultured in RPMI 1640 containing 10% FBS. White blood cells were isolated from peripheral blood of BALB/c mice using ACK lysis buffer (Sigma), and neutrophils were identified by flow cytometry as SSC-A^{high} (SSC stands for side scatter) GR-1^+, CD14^-, CD3^- . Monocyte-derived dendritic cells (moDCs) were generated by differentiating CD14^+ monocytes from peripheral blood and culturing for 6 days in the presence of interleukin 4 (IL-4) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Miltenyi).

Analysis of glycan content of antibodies. The relative abundance of antibody glycan structures were quantified by capillary electrophoresis as previously described (28). Briefly, antibodies were digested with IDES (FabRICATOR; Genovis) to separate Fe and F(ab')2, and N-linked glycans were removed using peptide-N-glycosidase F (PNGase F) (New England Biolabs). Free glycans were labeled with 50 mM 9-aminopyrene-1,4,6-trisulfonic acid (APTS) in 1.2 M citric acid and 1 M sodium cyanoborohydride in tetrahydrofuran (THF), and excess dye was removed using a size exclusion resin. Labeled glycans were loaded onto a 3130 XL ABI DNA Sequencer using a POP7 polymer in a 36-cm capillary. Peaks of 19 substructures were identified, and the relative abundance of a given structure was determined by calculating the area under the curve for each peak divided by the total area of all peaks.

Filoivirus challenge studies in mice. (i) Murine EBOV model. The lethal mouse-adapted EBOV mouse model was developed at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), using adult mice by serial passages of EBOV (Zaire) in progressively older suckling mice (29, 30). This model has been thoroughly validated (31). Female BALB/c mice, aged 6 to 8 weeks, were purchased from Charles River Laboratory. Upon arrival, mice were housed in microisolator cages and provided chow and water ad libitum. On day 0, mice were transferred to a biosafety level 4 containment area and challenged intraperitoneally with a target of 1,000 PFU of mouse-adapted EBOV. In two independent experiments, groups of mice (5 or 10 mice per group) were treated intraperitoneally with a range of antibody doses as indicated in the legend to Fig. 6. Control mice were simultaneously challenged but given only PBS at the corresponding treatment intervals. Mice were weighed as groups and monitored daily for 28 days postinfection. When experimental conditions in independent experiments were the same, the data for those groups were pooled for graphic presentation in figures and statistical analysis.

(ii) Murine SUDV model. The SUDV mouse model was developed at the USAMRIID utilizing the IFN-α/βR^−/− mouse model (mice lacking receptors for alpha interferon [IFN-α] and IFN-β) (32). IFN-α/βR^−/− mice (B6.Bd122(Sjfu1tm1m1g/Mjnaex), aged 8 to 10 weeks, on the C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME) and used for all SUDV challenge experiments. Upon arrival, mice were housed in microisolator cages and provided chow and water ad libitum. On day 0, mice were transferred to a biosafety level 4 containment area and challenged intraperitoneally with a target of 1,000 PFU SUDV-Boniface virus. Groups of mice (10 mice per group [5 male, 5 female]) were treated intraperitoneally with a range of doses of each antibody as indicated. Control mice were simultaneously challenged but given only PBS at the corresponding treatment intervals. Mice were weighed as groups and monitored daily for 28 days postinfection.

Animal research was conducted under a protocol approved by the U.S. Army Medical Research Institute of Infectious Diseases Institutional Animal Care and Use Committee (IACUC) in compliance with the Animal Research Act of 1996.
Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. The USAMRIID facility is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International and adheres to the principles stated in the Guide for the Care and Use of Laboratory Animals (26). Challenge studies were conducted under maximum containment in an animal biosafety level 4 facility. Animal studies were performed in a blind manner (personnel assessing results were unaware of which animals received which treatment). Animals were not specifically allocated or randomized into groups. The number of mice to be used in these studies was selected to measure and determine differences in levels of protection elicited by the different MAb treatments. Experience with the use of various analyses for determining the probability of differences between control and experimental mouse groups indicates the need for 5 to 10 mice per group. For lethality studies, a statistical difference between 0% in the control and 30% in the treated groups can be demonstrated using 10 mice per group with 90% power using a two-sided alpha level of 0.05. In the cases where large numbers of antibodies were tested in preliminary studies, five mice per group were tested, and then the results were confirmed with the larger group of 5 or 10 mice. In these cases, the results of the combination of both studies are shown. To demonstrate statistical differences in the treatment groups, data were analyzed using GraphPad software, version 6 (La Jolla, CA) using the Mantel-Cox log rank test and confirmed using the Gehan-Breslow-Wilcoxon test.

RESULTS

Antibody generation. To drive the generation of broadly reactive antibodies, BALB/c mice were immunized with a mixture of purified, engineered glycoprotein (GP) ectodomains lacking the highly divergent mucin-like domain (MLD) (GPΔmuc) for EBOV (Mayinga), SUDV (Boniface), and MARV (Musoke) using two different immunization strategies (Fig. 1A). Mice were also boosted intravenously with the same protein cocktail (without adjuvant) 1 to 3 days before harvesting spleens for fusion. Total IgG and neutralizing responses against the three filovirus GPs were determined in terminal bleeds (Fig. 1B and C). Splenocytes were harvested and fused to generate hybridomas.

More than 1,300 clones were initially screened for binding to the GP ectodomain (GPΔTM) for EBOV, SUDV, and MARV. As expected, the majority of the reactive clones were specific for a single GP species and were excluded from further analysis. A single MAb (m21D10) reactive to all three GP molecules and six clones reactive to both EBOV and SUDV but nonreactive to MARV were identified and grown for further analysis (Fig. 1A). Upon secondary screening, four cross-reactive but ebolavirus-specific clones (m16G8, m8C4, m17C6, and m4B8 [the “m” prefix indicates the murine origin of the antibody]) exhibited greater strength and breadth of binding. These four clones along with m21D10 were further subcloned to ensure clonal purity, and the MAbs were produced, purified, and analyzed for binding to GPΔTM from EBOV, SUDV, BDBV, RESTV, and MARV. The MAbs displayed various degrees of binding to most species of ebolavirus, but none, other than m21D10, bound to MARV, con-
consistent with the primary screening results (Fig. 2). The monoclonal antibody m16G8 bound moderately to BDBV but with low affinity to EBOV, SUDV, and RESTV glycoproteins (Fig. 2A). m8C4 showed preferential binding to SUDV and EBOV and to a lesser extent to BDBV but failed to bind to RESTV (Fig. 2B). m17C6 displayed strong binding to EBOV but exhibited lower levels of binding to SUDV and RESTV and very poor binding to BDBV (Fig. 2C). The strongest and most balanced binding was displayed by m4B8, which bound to the four ebolavirus species at low nanogram/milliliter concentrations (Fig. 2D). The only MARV-reactive clone, m21D10, exhibited the highest binding for BDBV and RESTV, followed by MARV and EBOV, and displayed a low level of binding to SUDV (Fig. 2E), thus representing a pan-filovirus antibody. Subtype-specific ELISA showed that m16G8 is an IgG2a, while the other MAbs are IgG1 (data not shown).

Characterization of the antibodies. None of the four pan-ebolavirus MAbs detected GP under denaturing conditions (data not shown), suggesting that they react with discontinuous epitopes. To determine the general binding regions of the conformational antibodies, we compared the binding of the MAbs to several EBOV GP constructs containing various functional domains of the protein. EBOV GP consists of the disulfide-bound GP1 and GP2 subunits that are responsible for receptor binding and membrane fusion, respectively (Fig. 3A). GP1 forms a chalice consisting of the receptor binding region (RBR) and the glycan cap positioned at the rim of the chalice as well as a C-terminal, highly glycosylated and disordered mucin-like domain (Fig. 3A) (20). GP2 wraps around GP1, and along with the N terminus of GP1, forms the base of the chalice. The unedited EBOV GP gene encodes soluble GP (sGP), which consists of amino acids 31 to 295, followed by a unique C-terminal tail (33, 34). sGP includes the glycan cap but lacks the MLD and GP2. During viral entry, GP undergoes cleavage by cathepsins at the N terminus of the glycan.
cap to generate cleaved GP (GPcl), representing truncated GP1 lacking both the glycan cap and MLD, which remains associated with GP2. We used GP\textsubscript{H9004}\,TM, GP\textsubscript{H9004}\,muc, GPcl, and sGP to systematically explore the binding region of the four pan-ebolavirus MAbs. As shown in Figure 3B to E, m4B8 and m17C6 bound to all four GP forms, indicating that the respective conformational epitopes are in GP1 between amino acids 31 to 200. In contrast, m16G8 failed to bind to sGP (Figure 3E), and since its binding was not dependent on MLD (Figure 3B and C), it is likely to have contact points in GP2. Its relatively poor binding to GPcl suggests that its epitope could be affected by enzymatic cleavage. The m8C4 MAb bound to all GP forms except for GPcl (Figure 3D), suggesting that its epitope lies within the glycan cap or the cathepsin cleavage site itself.

In contrast to the pan-ebolavirus MAbs, m21D10 reacted with chemically denatured antigen (data not shown) and recognized GP from EBOV, SUDV, BD BV, RESTV, and MARV in Western blots (Figure 4A), indicating that it binds to a continuous epitope. To define the linear epitope of m21D10, we used a library of 15-residue overlapping peptides spanning the entire GP from EBOV, SUDV, and MARV to search for peptides that specifically compete with the binding of m21D10 to GP. This competition assay (Figure 4B and C) revealed that m21D10 binds to residues 81 to 90 of EBOV and SUDV, as well as positions 65 to 74 of MARV GP. This stretch of amino acids is located within the RBR, corresponds to α1β4 of EBOV GP (20), and is highly conserved across filoviruses (Figure 4C and D). This epitope appears to be heavily concealed on the surface of GP, as removal of the MLD significantly enhanced the binding of m21D10 to EBOV, SUDV, and MARV GP (Figure 4E and F). The binding of m21D10 to GPcl was exponentially higher than its binding to GP\textsubscript{H9004}\,muc (EC\textsubscript{50} reduced >1 log unit), indicating that the glycan cap also restricts access to this site (Figure 4F).

Using competition ELISA, the pan-ebolavirus MAbs were tested for possible epitope overlaps among each other or previously described anti-EBOV MAbs KZ52 (21) and 13C6 (35, 36) (Table 1). KZ52 binds at the base of the GP chalice (20) to an epitope shared by ZMapp components 2G4 and 4G7 (19), while 13C6, another component of ZMapp (14), is a glycan cap binder (19). m16G8 did not show significant competition with any of the four pan-ebolavirus MAbs, KZ52, or 13C6, suggesting that it recognizes a unique epitope with little steric interference by the other MAbs tested. The m8C4 MAb that likely binds the glycan cap or the cathepsin cleavage site itself.

Virus neutralization activity. The MAbs were tested for neutralization activity.
neutralization of EBOV and SUDV using GFP-expressing VSV pseudotyped with EBOV or SUDV GP (27). Out of the MAbs tested, only m8C4 showed appreciable neutralizing activity in this system. As shown in Fig. 5A, m8C4 effectively inhibited both EBOV GP-VSV and SUDV GP-VSV with IC50 values of about 1.5 and 0.75 μg/ml, respectively. The neutralizing activity of the MAbs was then tested on live virus using a high-content imaging-based assay. Consistent with the VSV pseudotype assay, only m8C4 effectively neutralized both SUDV (Fig. 5B) and EBOV (Fig. 5C). The neutralization potential of m21D10 was also tested against EBOV, SUDV, and MARV using the VSV pseudotype system, but this antibody exhibited no neutralizing activity at up to 100 μg/ml (data not shown).

Efficacy in the mouse model of EBOV infection. The efficacy of the MAbs was tested in BALB/c mice using mouse-adapted EBOV (MA-EBOV) (29). Groups of mice were challenged with
target dose of 1,000 PFU of MA-EBOV, followed by two intraperitoneal injections of antibody (25 mg/kg of body weight) at 2 and 3 days postinfection. As shown in Fig. 6A, using this regimen, all mice treated with m4B8 and 7 out of 15 mice receiving m8C4 survived the lethal challenge, while m17C6 and m16G8 provided only 20% and 13% protection, respectively. In contrast, all 15 control mice as well as mice treated with m21D10 succumbed to infection within 5 to 9 days. Control mice and animals treated with m21D10 lost more than 25% weight before dying, while all groups of mice treated with pan-ebolavirus MAbs showed considerably less weight loss (Fig. 6B). Given the strong protection afforded by two injections of m4B8, we examined whether delayed treatment with this antibody would provide protection. When treatment with m4B8 was delayed until 3 days after infection, 80% of the mice survived the challenge (Fig. 6C) with less than 18% average weight loss (Fig. 6D).

The success of the EBOV-specific cocktails ZMab (18), MB-003 (11), and ZMapp (14) has suggested that antibodies targeting multiple distinct epitopes may act synergistically and have greater therapeutic efficacy. To this end, we examined whether the efficacy of m8C4 (binding near or on the glycan cap) can be enhanced by m16G8 (an antibody likely to bind GP2). When mice were treated with a cocktail of the two antibodies at 15 mg/kg, 80% of the animals survived the challenge (Fig. 6C) with less than 18% average weight loss (Fig. 6D).

Efficacy in a SUDV infection model. A lethal mouse model for SUDV has been recently developed using mice lacking receptors for IFN-α and IFN-β (IFN-α/βR−/−) (32). Since m8C4 showed strong neutralization toward SUDV, as a proof of concept, we sought to test this antibody in the SUDV mouse model. Groups of 10 mice were infected with 1,000 PFU of wild-type SUDV. One group received m8C4 (10 mg/kg) 24 h before and 24 h and 72 h after infection, while a second group received m8C4 (5 mg/kg) only on day 1 postinfection. While all control mice and mice receiving a single 5-mg/kg dose of m8C4 succumbed to infection, treatment with three doses of m8C4 led to 70% protection and lower and delayed weight loss (Fig. 7A and B). Thus, m8C4 represents the prototype of an antibody with cross neutralization and protective efficacy against two widely divergent ebolavirus species.

Effector functions mediated by pan-ebolavirus antibodies. Antibody-mediated activation of innate immune cell effector functions, including phagocytosis and cytotoxic activity mediated by neutrophils, monocytes, and NK cells, can play a critical role in protection from viral and bacterial infections (37). Thus, to explore the role of nonneutralizing functions in antibody-mediated protection, antibodies were evaluated for their ability to rapidly recruit phagocytic clearance of EBOV-specific immune complexes by distinct innate immune cells. Using the murine RAW phagocytic cell line, potent phagocytosis was observed for all MAbs with the exception of m16G8 (Fig. 8A). Notably, only m4B8, and to a lesser extent m8C4, induced appreciable phagocytosis using the human monocytic THP-1 cells (Fig. 8B). Similar to

TABLE 1 Binding competition between pan-ebolavirus MAbs, KZ52, and 13C6a

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<th>Antigen</th>
<th>Detecting antibody</th>
<th>% binding competition between pan-ebolavirus MAbs</th>
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<td>EBOV GP</td>
<td>m8C4-biotin</td>
<td>m8C4 1 m16G8 11 h17C6 8 h4B8 58 KZ52 59 c13C6 59</td>
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a Competition ELISA was performed as described in Materials and Methods. To enable detection of the antibodies in the presence of each other, some antibodies were biotinylated (m8C4 and KZ52), while some were used as humanized (h4B8 and h17C6 [h stands for humanized]) or mouse-human chimeric forms (c13C6 [c stands for chimeric]).

FIG 5 Neutralizing activity of the pan-ebolavirus MAbs against EBOV and SUDV. (A) Percent neutralization by m8C4 of vesicular stomatitis virus (VSV) pseudotyped with EBOV or SUDV GP. (B and C) Neutralizing activity of the pan-ebolavirus antibodies against wild-type SUDV (B) and EBOV (C) determined using a high-content imaging-based neutralization assay as described in Materials and Methods.
the RAW cells, m4B8, m17C6, and m8C4 induced robust neutrophil-mediated phagocytosis; however, m16G8 mediated negligible activity (Fig. 8C). Finally, m4B8 and m8C4 induced higher phagocytosis by human monocyte-derived dendritic cells (moDCs) than m17C6 and m16G8 did (Fig. 8D). Interestingly, phagocytic activity was associated with lower galactosylation, reduced fucosylation, and enhanced addition of bisecting N-acetylglucosamine (GlcNAc) glycan structures (Fig. 8E), which have previously been associated with Fc effector functionality (38, 39). These data demonstrate that different Fc effector functional profiles exist among the MAbs, including highly phagocytic antibodies (m4B8 and m8C4) that induce robust phagocytosis by all innate immune cells tested, antibodies that induce selective phagocytosis in particular cell types (m17C6), and antibodies that do not induce phagocytosis at all (m16G8), and suggest that unique Fc effector profiles may be important for understanding the underlying mechanisms of antibody-mediated protection from EBOV infection.

DISCUSSION

The 2014–2015 outbreak of EVD in West Africa has highlighted the threat of filoviruses to global health (40). While significant progress has been made toward vaccines and immunotherapeutics for EBOV, the development of therapeutics against other filovirus species has been lagging behind. Broadly protective treat-
ment modalities are urgently needed, as the nature of future outbreaks cannot be predicted.

Here we report novel murine monoclonal antibodies cross-reactive to four species of ebolavirus and a pan-filovirus antibody. The only ebolavirus species that was not tested in this study is TAFV, which has caused disease in only one nonlethal human case (41). However, the TAFV GP shows 90% sequence identity with BDBV in regions excluding the MLD, and thus, these antibodies are very likely to bind to TAFV.

We applied an immunization strategy in mice using a cocktail of engineered glycoproteins from three filovirus species lacking the highly divergent MLD to drive immune responses to conserved epitopes. More than 1,300 clones had to be screened to select four MAbs with broad ebolavirus reactivity and a single pan-filovirus MAb, indicating that such antibodies are very likely to bind to TAFV.

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MAb therapy has yet to be demonstrated. Nonetheless, our results suggest that effective pan-ebolavirus therapeutic antibodies can be developed.

Another MAb described here, m4B8, binds with high affinity to EBOV, SUDV, BDBV, and RESTV and provides 100% efficacy after two injections in a mouse model of EBOV infection. Single delivery of this MAb on day 3 (peak of viremia in mice [29]) also provided significant protection. While m16G8 and m17C6 provided low levels of protection in mice, consistent with the current paradigm emerging from studies with ZMapp [14], a cocktail of m8C4 and m16G8 displayed synergy when combined at lower doses.

Previous results indicated that the most effective EBOV antibodies target the glycan cap (13C6) and the base of the trimeric GP chalice (4G7, 2G4, and KZ52) [14, 19]. Since the KZ52 epitope appears to be strictly species restricted, it is significant that none of the pan-ebolavirus MAbs reported here bind the KZ52-type base epitope, suggesting that there are additional epitopes that can be exploited for development of broadly protective antibodies. On the other hand, there may be additional epitopes on or surrounding the glycan cap that can be exploited. For example, m8C4 binds to a conformational epitope that appears to involve the glycan cap and possibly neighboring residues within the core GP1 and -2 domains.

The most protective cross-reactive MAb, m4B8, binds to a conformational epitope within the first 170 amino acids of mature GP1. While the details of this epitope remain to be defined by crystallographic analysis, it appears to be a novel epitope, as m4B8 did not compete with any known antibodies tested in our study. Interestingly, m4B8 did not show neutralizing activity. It is possible that in vitro neutralization assays may not be sensitive enough to detect modest neutralizing activities, as in plaque reduction assays, we did observe low levels of neutralization by m4B8 (data not shown). Alternatively, the primary mechanism of action of m4B8 may involve Fc-dependent effector mechanisms. Indeed, our studies show that m4B8 was highly effective in mediating phagocytosis by neutrophils, monocytes, and dendritic cells.

The RBR shows high degree of homology among filoviruses. Contrary to our expectation, only a single MAb (m21D10) emerged from our screen that bound to RBR. This antibody bound poorly to EBOV GP ectodomain or virus-like particles (data not shown), but the binding was enhanced exponentially upon proteolysis at the cathepsin cleavage site, suggesting that access is restricted by MLD and glycan cap. Recently, Flyak et al. isolated several related MAbs from a human survivor of MARV infection [47] with various degrees of reactivity to EBOV GP. Co-crystal structure of one of these MAbs (MR78) showed that it binds to the putative receptor binding site of MARV GP [24]. The linear epitope for m21D10 is distinct from the MR78 epitope, but it does share several contact residues (Fig. 9). This epitope includes MARV GP F72 (equivalent to EBOV F89, which appears to be part of the receptor binding site [20, 24]) and R73 (equivalent to EBOV R90), which makes contacts with the fusion loop of the adjacent GP molecule (Fig. 9) in both MARV [24] and EBOV [20] GP structures. Unlike MR78-type antibodies, m21D10 did not neutralize MARV, possibly due to differences in sequence or accessibility of the site. It is possible that an affinity matured version of m21D10 may overcome the restricted access to the RBR.

In addition to neutralization, antibodies can provide protection following infection through the recruitment of Fc-dependent innate immune effector functions. Interestingly, three of the four pan-ebolavirus antibodies induced Fc-dependent phagocytosis in multiple innate immune cells, linked to a glycan profile marked by lower galactosylation, lower fucosylation, and increased bisecting GlcNAc addition. Fc effector functionality is also modulated by subclass, with IgG2a and IgG2b being the most functional subclasses of mouse antibodies, based on Fcy receptor (FcyR) interactions [48]. Interestingly, despite m16G8 being IgG2a, whereas the other three antibodies are IgG1, m16G8 still exhibited reduced function compared to the other antibodies.

Fc-mediated functionality and neutralization are not mutually exclusive activities, and recent data using HIV-specific neutralizing antibodies suggest that many neutralizing antibodies depend on Fc effector functions to mediate their in vivo protective activity.
highlighting the importance of recruiting Fc-mediated innate immune functions, even in the presence of potent neutralization. Thus, while it is likely that while direct virus neutralization by m8C4 significantly contributes to the protection observed in vivo, this antibody may also utilize Fc effector functions to mediate protective immunity.

In summary, we have discovered novel pan-ebolavirus conformational epitopes that can be targeted to generate effective antibody cocktails against multiple ebolaviruses. Both novel and cross-reactive antibodies are needed, as current cocktails are reactive only against EBOV, and two of the three components of ZMapp overlap with each other at the base. These results provide a basis for further testing of synergistic pan-ebolavirus cocktails in NHPs. Furthermore, these broadly reactive antibodies represent invaluable tools for development of diagnostics for filoviruses.

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Research was conducted under an IACUC-approved protocol in compliance with the Animal Welfare Act, PHS policy, and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Accreditation and Certification of Laboratory Animal Care International and adheres to the principles stated in the 2011 Guide for the Care and Use of Laboratory Animals. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

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REFERENCES


