Delayed treatment of Ebola virus infection with plant-derived monoclonal antibodies provides protection in rhesus macaques

Gene Garrard Olinger, Jr.*1,2, James Pettitt*1,3, Do Kim4, Cara Working5, Ognian Bohorov6, Barry Bratcher2, Ernie Hiatt2, Steven D. Hume7, Ashley K. Johnson2, Josh Morton2, Michael Pauluy8, Kevin J. Whaley9, Cali M. Lear3, Julia E. Biggins9, Corinne Scully9, Lisa Hensley9,3 and Larry Zeitzlin9,2

*Division of Virology, United States Army Medical Research Institute of Infectious Diseases, Frederick, MD 21702; 1Mapp Biopharmaceutical, Inc., San Diego, CA 92121; and 2Kentucky BioProcessing, LLC, Owensboro, KY 42301

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Filovirus infections can cause a severe and often fatal disease in humans and nonhuman primates, including great apes. Here, three anti-Ebola virus mouse/human chimeric mAbs (c13C6, h-13F6, and c6D8) were produced in Chinese hamster ovary and in whole plant (Nicotiana benthamiana) cells. In pilot experiments testing a mixture of the three mAbs (MB-003), we found that MB-003 produced in both manufacturing systems protected rhesus macaques from lethal challenge when administered 1 h postinfection. In a pivotal follow-up experiment, we found significant protection (P < 0.05) when MB-003 treatment began 24 or 48 h postinfection (four of six survived vs. zero of two controls). In all experiments, surviving animals that received MB-003 experienced little to no viremia and had few, if any, of the clinical symptoms observed in the controls. The results represent successful postexposure in vivo efficacy by a mAb mixture and suggest that this immunoprotectant should be further pursued as a postexposure and potential therapeutic for Ebola virus exposure.

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or more than 35 y, a therapy has been sought to treat the severe lethal disease caused by Ebola virus (EBOV; family Filoviridae), which are among the most virulent infectious agents known, causing acute and frequently fatal hemorrhagic fever in humans and nonhuman primates (NHP) (1, 2). Outbreaks in humans occur intermittently, causing localized high morbidity and mortality. Due to its infectiousness, the lack of approved diagnostics, and the rapidity of modern travel, the potential exists for any outbreak to become an international epidemic. Currently there are no licensed vaccines or treatments against EBOV infection. Candidate postexposure interventions in advanced development include siRNA and phosphorodiamidate morpholino oligomers (PMOs) antisense strategies, as well as postexposure immunization with a vesicular stomatitis virus (VSV)-based vaccine (3, 4). Hurdles to the adoption of convalvalescent-phase serum as a medical countermeasure are likely cost-prohibitive and would have significant logistical and regulatory hurdles. As an approach to develop a mAb-based medical countermeasure, Marzì et al. (5) demonstrated that two neutralizing mouse/human chimeric mAbs against EBOV could provide limited protection in rhesus macaques (one of three animals survived) when dosing was initiated 24 h before challenge (1,000 pfu 1LD50). More recently, Qi et al. (6) found a mixture of neutralizing murine mAbs could protect cynomolgus macaques when given 1 (four of four survived) or 2 d p.i. (two of four survived).

Development of the mAb-based EBOV immunoprotectant MB-003 was built upon prior work characterizing three mouse mAbs (13C6, 13F6, and 6D8) directed against three distinct, nonoverlapping EBOV glycoprotein (GP) epitopes (14). Only one of these mAbs (13C6) binds to secreted GP (sGP), which has been speculated to act as a decoy for protective antibodies (22). Two of these mAbs (13C6 and 6D8) neutralize virus in the presence of complement, and one has no neutralizing activity (13F6). These mAbs were shown to individually protect against lethal challenge prophylactically in mouse models of EBOV infection and have a therapeutic window of at least 48 h after viral exposure (14). To develop a product that would be appropriate for human use, the murine mAbs were deimmunized (23) and/or chimerized with human constant regions, yielding c13C6, h-13F6, and c6D8. To evaluate a more cost-effective and scalable alternative to production in Chinese hamster ovary (CHO) cells, the mAbs were produced in a Nicotiana benthamiana-based rapid antibody manufacturing platform (RAMP) using magnICON (ICON Genetics) deconstructed viral vectors (24). The RAMP system allows rapid, scalable production of mAbs in less than a month, and has been used to produce mAbs under cGMP conditions (25). Via the use of a transgenic strain of N. benthamiana in which plant-specific glycosyltransferases (α1,3 fucosyltransferase and β1,2 xylosyltransferase) are inhibited by RNAi (26), the RAMP-
derived mAbs have homogenous mammalian glycans. In the mouse-adapted Ebola model, the RAMP mAbs provided protection superior to CHO-derived mAbs, likely due to the increased antibody-dependent cellular cytotoxicity (ADCC) activity conferred by the N-glycans lacking core fucose present on the fragment crystallizable (Fc) region (23).

Here, we report the efficacy of CHO and RAMP-derived MB-003 mixture (MB-003\textsubscript{CHO}–MB-003\textsubscript{RAMP}, respectively) using the lethal EBOV NHP model. The rhesus macaque model was chosen because symptom onset more closely parallels human disease progression compared with the cynomolgus model (27). This proof-of-concept study presents data demonstrating that the plant-produced MB-003\textsubscript{RAMP} is efficacious in preventing lethal disease in EBOV-infected macaques when administered 24 or 48 h after virus challenge.

Results

Antibody Analysis. Analysis of the CHO-derived mAbs indicated core fucosylated nongalactosylated (GnGnF) and monogalactosylated (AGnF) N-glycan structures were the major glycoforms (Fig. 1). In contrast, no major core fucosylated structures were detected in the RAMP-derived mAbs (Fig. 1). These RAMP mAbs carried a single major biantennary N-glycan with terminal GlcNAc on each branch—namely, GnGn—corresponding to a fucose-free form of one of the major glycoforms found in the CHO-produced mAbs (GnGnF). EBOV glycoprotein antigen-binding ELISAs and mouse efficacy testing performed as part of the release testing (i.e., potency assays) for the mAbs demonstrated binding capability indistinguishable between the CHO- and RAMP-derived mAbs.

Clinical Observations and Outcomes. In the first pilot study (Table 1), macaques were challenged i.m. with 100 pfu EBOV, and treatment (n = 2) was initiated 1 h p.i. with MB-003\textsubscript{CHO} (50 mg kg\textsuperscript{-1} mAb\textsuperscript{-1}). Animals received an additional dose on days 4 and 8. MB-003\textsubscript{CHO}-treated animals displayed no evidence of infection, and no virus was detected in serum by RT-PCR. In contrast, the two control animals treated with PBS or irrelevant control mAb (Symagis; MedImmune) displayed symptoms of infection and subsequently died (days 7 and 9, respectively).

In a follow-up pilot study, the challenge was increased to 1,000 pfu, and treatment was again initiated 1 h p.i. (with additional dosing on day 4 and 8). Macaques received either MB-003\textsubscript{CHO} (50 mg kg\textsuperscript{-1} mAb\textsuperscript{-1}) or MB-003\textsubscript{RAMP} (16.7 mg kg\textsuperscript{-1} mAb\textsuperscript{-1}), and the control animal received PBS. The difference in dosing between CHO- and RAMP-derived mAbs was based on murine studies showing a threefold improvement in potency of the RAMP-derived mAbs compared with the CHO-derived mAbs (23). One of two macaques treated with MB-003\textsubscript{CHO} and three of three treated with MB-003\textsubscript{RAMP} (P < 0.02 vs. historical controls) survived challenge (Table 1) and had no detectable virus by plaque assay and RT-PCR. One CHO-treated animal was euthanized on day 12 when the clinical score surpassed euthanasia criteria. The time to death was within the range seen historically with the stock used for challenge, and the pathology report concluded that findings appeared consistent with fibrillogenic infection. This animal displayed decreased platelets and glucose levels and increased aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. The PBS control animal became clinically ill (Table 1) but recovered and displayed a transient drop in platelets and a transient rise in AST. This control animal and the nonsurviving MB-003\textsubscript{CHO}-treated primate both showed significant levels of virus from serum as detected by RT-PCR.

Testing in the pivotal study was confined to RAMP-derived mAbs due to their superior efficacy to CHO-derived mAbs in murine studies (23) and the suggested superiority in Study 2 (e.g., 100% protection vs. 50% with threefold less MB-003). Initiation of treatment with the MB-003\textsubscript{RAMP} (16.7 mg kg\textsuperscript{-1} mAb\textsuperscript{-1}) 24 or 48 h p.i. (1,000 pfu ~1,000 LD\textsubscript{50}) was tested using a different viral stock (Fig. 2A). Animals received three additional MB-003\textsubscript{RAMP} doses (days 5, 8, and 10 for the 24-h p.i. group and days 6, 8, and 10 for the 48-h p.i. group). Four of the six animals treated (two from each group) survived challenge (P < 0.05 for both groups vs. historical controls) challenged with this viral stock and P < 0.05 for the 48-h group against the two internal controls; Fig. 2C). Further, these animals displayed no signs of illness and no significant changes in platelet count, glucose, or liver enzyme levels (Fig. 3A–D). These animals had virus detected in serum by RT-PCR at 1,000-fold less than controls (Fig. 3E). One 24-h p.i.-treated animal was found dead on day 11, and one 48-h p.i.-treated animal was euthanized on day 16 when the clinical score surpassed euthanasia criteria (Fig. 2D). These two animals displayed signs of illness (Table 1) and had blood chemistry changes, but at reduced scale from controls. Viral levels in serum detected via RT-PCR in these animals were 1,000-fold less than controls (Fig. 3C). Both control animals (one received an irrelevant RAMP-derived mAb 24 h p.i., and the other received PBS 48 h p.i.) succumbed to infection on day 7. These two animals had dramatic drops in platelet and glucose levels, substantial increases in enzyme levels, and high viral titers determined by RT-PCR and viral culture (Fig. 3). Pathology findings for all four nonsurviving animals were consistent with filovirus infection.

Discussion

There is an urgent need for a cost-effective postexposure therapeutic to treat infection and halt transmission during EBOV outbreaks, as well as for use in the event of a bioterror threat. The importance of antibodies in the adaptive immune response during vaccination is well recognized, and the use of antibodies as therapy against infectious disease is building credibility as well (6, 28, 29). It is only recently, however, that passive immunization (or EBOV—with macaque polyclonal IgG and with mouse mAbs—that has been demonstrated (20, 21). The results presented here further extend these findings to a mixture of mAbs appropriate for human administration. The evaluation of protection using MB-003 in the macaque model shows a system in which both the pathogenesis of disease and immune responses of the animal host imitate the human condition. The mixture of three mAbs in a postexposure treatment regimen against EBOV was designed to mimic a potential needle-stick scenario in a laboratory setting.

Although involving small numbers, the results from the first two pilot studies were informative. In the first study, MB-003\textsubscript{CHO} protected the two treated animals from a lethal 100 pfu challenge. These animals were completely protected from morbidity, in contrast to the two controls that displayed clinical illness and succumbed to infection. In the second experiment, MB-003 from the CHO and RAMP system were compared, using a more rigorous challenge of 1,000 pfu. Results from this experiment are confounded by the survival of the control, which has occurred one other time in the last 2 y with this viral stock (of 15 animals total; given the likely genetic diversity of these wild-caught macaques, it is not entirely surprising that controls occasionally can survive, as do humans, perhaps due to differences in CD4+, CD8+, or IFN-γ responses) (30). Nevertheless, the control did show symptoms of infection and viremia. The protection conferred by MB-003\textsubscript{RAMP} (three of three protected; P < 0.02 compared with historical
controls), administered at one-third of the dose of MB-003, protected animals (one of two protected), is consistent with our findings in mice that the RAMP-derived mAbs have superior potency (22). Because the only difference between the CHO and RAMP mAbs is their glycosylation, this would suggest a significant role for glycan-dependent Fc receptor-mediated effector functions in the protection conferred by these mAbs. Indeed, the absence of core fucose on IgG1 is known to increase binding of mAb to FcγRIII, resulting in a dramatic improvement in ADCC activity compared with antibody with core fucose. These results in total suggest that ADCC plays a critical role in the protective efficacy conferred by the MB-003 mAbs. It is interesting to speculate whether the incomplete or absence of protection previously observed with polyclonal and monoclonal antibodies of high neutralizing activity (17–19, 31) may have been due to relatively reduced ADCC activity conferred by their glycosylation—CHO cells, and mammalian cells in general, produce only a small percentage of IgG lacking core fucose.

In our final experiment, four of six animals treated 1 or 2 d p.i. with MB-003RAMP survived challenge with a historically 100% lethal viral stock (n = 6), whereas both controls died. The two treated animals that succumbed to challenge experienced either hindered or delayed course of disease. Because c13C6 and c6D8 neutralize EBOV in the presence of complement (14), the inability to detect virus in serum by plaque assay in these two animals (Fig. 3A), confirmed that the two mAbs in MB-003 have not been tested individually in macaques. Elimination of an ineffective mAb from the mixture and/or higher dosing could improve efficacy. Alternatively, because ADCC is an important mechanism of action for these mAbs (23), polymorphisms of the ADCC-mediating FcγRIIIa receptor (28, 32) within the macaque population used in the studies could have affected mAb potency. A polymorphism in approximately half of humans (32) has been shown to affect ADCC and tumor-cell killing by the FDA-approved mAb, rituximab (33). Although less well-characterized, three FcγRIII polymorphisms have been reported in macaques (34, 35), and in one study, 33% (n = 9) of macaques had a FcγRIII polymorphism (35). Finally, there may have been an as-yet-uncharacterized variability within the study population that affected the host response to infection.

In this study we used a well-characterized EBOV source (Zaire Kikwit). With improved sequencing tools, the role of EBOV isolate sequence differences has recently become topical (36). For instance, the sequence of EBOV used in this study appears to contain an additional uracil residue in the GP gene-editing site. If this is a true mutation (the site causes stuttering of polymerases and may affect the function of sGP) is unknown, sGP has been hypothesized to act as a decoy for protective antibodies. One of the three MB-003 mAbs does bind to sGP (13C6), and if this mAb alone is providing the protective efficacy observed in the studies described here, the mAb

### Table 1. Clinical events on days 1–28 post-EBOV challenge

<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Finding</th>
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<tbody>
<tr>
<td>Study 1</td>
<td>1A (S)</td>
<td>1 h p.i. CHO</td>
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<td></td>
<td>2A (S)</td>
<td>1 h p.i. CHO</td>
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<td></td>
<td>3A (NS, day 9)</td>
<td>1 h p.i. mAb control</td>
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<td></td>
<td>4A (NS, day 7)</td>
<td>1 h p.i. PBS control</td>
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<td>Study 2</td>
<td>1B (S)</td>
<td>1 h p.i. CHO</td>
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<td></td>
<td>2B (NS, day 12)</td>
<td>1 h p.i. CHO</td>
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<tr>
<td></td>
<td>3B (S)</td>
<td>1 h.p.i. RAMP</td>
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<td>4B (S)</td>
<td>1 h.p.i. RAMP</td>
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<td>7C (S)</td>
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<td></td>
<td>8C (NS, day 7)</td>
<td>48 h.p.i. PBS control</td>
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*Thrombocytopenia is defined as ≥35% decrease in platelets. Petechial rash is defined as 10–40% of body surface area; significant petechial rash is defined as >40% of body surface area. ↑↑↑, two- to threefold increase; ↑↑, four- to fivefold increase; ↑↑↑, fivefold increase; ↑, two- to threefold decrease. BUN, blood urea nitrogen; GGT, γ-glutamyl transpeptidase GLU; glucose; NS, nonsurvivor; S, survivor; TBIL, total bilirubin.

*Euthanized by Veterinary Medicine Division staff (viremic via plaque assay).
may have reduced efficacy against a strain without this mutation. However, 13C6 is highly protective in a mouse model that uses a mouse adapted strain (Zaire Mayinga) that lacks this mutation, which would argue against this concern. Regardless, testing of MB-003 against strains other than Zaire Kikwit is planned.

These proof-of-concept studies were designed to reflect post-exposure prophylaxis circumstances potentially seen in human cases, and show the potential for improvement upon the 1 h p.i. treatment window demonstrated with other EBOV products (siRNA, PMOs, and VSV) currently in advanced development. Additional studies will be performed with larger groups to determine if MB-003 has efficacy as a therapeutic (i.e., the treatment window extends to the onset of diagnosis). Future experiments will also be aimed at determining which of the mAbs or combination of mAbs used in the mixture offers optimal protection, and identifying the best treatment regimen. Stability studies are ongoing, and the RAMP-derived mAbs, like mAbs from mammalian cell culture, are expected to have a good stability profile, an important trait for drugs that may be included in the Strategic National Stockpile.

**Methods**

**Production of MB-003 mAbs in CHO.** Stable CHO cell lines were generated (38) expressing h-13F6, c13C6, and c6D8 mAbs and cultured in CD OptiCHO Medium (Invitrogen) supplemented daily with CHO Feed Bioreactor Supplement (Sigma). The CHO culture was grown in suspension using a WAVE Bioreactor 20/50 EHT System (GE Healthcare) equipped with a WAVEPOD

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Fig. 2. Initiation of treatment of rhesus macaques with MB-003 at 24 or 48 h postinfection provides protection against EBOV. Delayed treatment with MB-003RAMP (16.67 mg·kg$^{-1}$·mAb$^{-1}$) was given 24 h (purple arrows) and 48 h (green arrows) after being challenged i.m. with 1,000 pfu of EBOV. (A) After challenge (red arrow), treatments were given on days 1, 5, 8, and 10 for the 24-h group and the irrelevant anti-HIV RAMP mAb control; the 48-h group and PBS control were treated on days 2, 6, 8, and 10. (B) Dosing was verified by recombinant GP binding ELISA. (C) A survival curve was generated with experimental groups, corresponding controls, and historical controls from the last 2 y challenged with stock 22433. Animals were observed daily (D) and scored for the duration of the study post-challenge. The sudden spike in observational score for primate 5C was due to a seizure, and the animal was euthanized shortly thereafter. Open symbols represent animals that survived, and filled symbols represent animals that succumbed to infection. Blue symbols indicate treatment initiation 24 h p.i. and green symbols 48 h p.i.; red indicates controls.

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Fig. 3. Clinical analysis and viral titers for the duration of study. Changes observed on days 0, 3, 6, 10, 14, 21, and 28 (additional day of euthanasia measurement on day 16 for primate 5C; additional day 8 chemistry analysis for all primates) for (A) platelet counts; (B) glucose; (C) AST; and (D) ALT. (E) RT-PCR-derived genomic equivalents (ge) and (F) viral titer as calculated by agarose plaque assay. All samples were analyzed via RT-PCR and plaque assay. Error bars (too small of an error to be seen for most points) in E and F represent SD (n = 3). Open symbols represent animals that survived, and filled symbols represent animals that succumbed to infection. Blue symbols indicate treatment initiation 24 h p.i. and green symbols 48 h p.i.; red indicates controls.
Production of MB-003 mAbs in A. thaliana. For transient expression of the MB-003 mAbs in plants, we used the "magnification" procedure (24) with minor modifications as described previously (23). Briefly, plants grown for 24−26 d in an enclosed growth room at 22−24 °C were used for vacuum infiltration. Equal volumes of overnight-grown Agrobacterium cultures were mixed in infiltration buffer (10 mM Mes (pH 5.5) and 10 mM MgSO4), resulting in a 1:1 volume mixing individual culture. Using a 300-L custom-built (Kentucky Bioprocessing) vacuum chamber, the aerial parts of entire plants were inverted into the bacterial/buffer solution and a vacuum of 22 inches of mercury was applied for 2 min. At 7 d postinfiltration, leaf tissue was extracted with a Corenco double-stack disintegrator using a 0.5:1 buffer-to-plant tissue ratio (extraction buffer: 100 mM Tris, 40 mM acetic acid, 1 mM EDTA (pH 8.5)). The extract was adjusted to pH 8.0 with 10 M NaOH. Extract was clarified using a plate-frame filter press (FertelAslop) with 1.0-mm pads. The antibody was captured from the filtrate using a MabSelect SuRe (GE Healthcare) Protein A column. The column was equilibrated and washed with Tris running buffer and eluted with acetic acid. The resulting eluate was neutralized using 1 M Tris. The mAb solution was then further purified via Q filtration (Muplex Q membrane; Pall). The final polishing column for c13C6 and c6D8 was a CHX, 8% (wt/vol) mannitol, and 0.005% polysorbate 20 using tangential filtration buffer [10 mM Mes (pH 5.5) and 10 mM MgSO4]. The mAbs were fully as−80 °C until used. All puri−cation steps were performed using liquid chromatography−MS.

N-Glycan Analysis. N−linked glycans were released by digestion with N−glycosidase F (PNGase F) and subsequent derivatization of the free glycan with an hydroxylamine−trifluoroacetic acid (HPLC−MS).

N-Linked glycan structures were determined by HPLC−MS analysis. For each mAb, the N−linked glycan composition was determined by comparing the sample to theoretical glycan structures and standard glycan standards. The percentages of each glycan structure were determined by comparison with theoretical glycan structures and/or by comparing with the theoretical glycan structures. Confirmation of glycan structure was performed using liquid chromatography−MS.

Virus Stocks. EBOV (Zaire Kikwit strain) stocks were developed at the US Army Medical Research Institute of Infectious Diseases (USAMRIID) using virus originally isolated from an infected patient during the 1995 outbreak and passaged in Vero E6 cells. Stock material used in the first two studies was three passages from the original isolate (the first two of which were performed at the Centers for Disease Control) stock material used in the third study was four passages from the original isolate. This stock has been deemed the national stock for preclinical studies for advanced products. The stock was made by the Department of Defense Critical Reagents Program under the Joint Program Executive Office for Chemical and Biological Defense. The virus and future viruses generated under this program have been developed under the Filovirus Animal Non−Clinical Program, a multiple−agency group including the Food and Drug Administration to prepare for validated/ regulated clinical studies. Statistical comparisons between the stocks used here found no difference in the mean times to death (P = 0.7854) or in the survival curves of all historic controls (P = 0.7873).

NHP Challenge and Care. Adult male and female rhesus macaques (RHM) were caged individually. RHMs were placed in training jackets (Lomir Biomedical Inc.) for acclimatization at least 4 d before surgery. Surgeries to insert central venous catheters (Groshong 7F; Bard) were performed, and adequate re−cruitment for each mAb was given before transfer into BSL4 contamination. After catheter placement, custom jackets were used to house and protect the animals (Lomir Biomedical Inc.). These lines were flushed with PBS (BD) and locked with 3 mL heparin (BD) at least once every other day to maintain catheter func−tionality. Animals were attached via a mounted swivel system (Lomir Bio−medical Inc.) and acclimatized to containment −7 d before challenge. Animals were given monkey chow, primate treats, fruits, and vegetables throughout the course of the study. Animals were observed at least once daily to monitor overall health.

Before challenge, RHMs were anesthetized via i.m. injection of Telazol (0.03 mL/kg) and given a brief physical, at which time baseline weight and temperature were established. Animals were challenged via i.m. route with a target dose of 1,000 pfu/mL diluted from stock virus in DMEM; the actual dose of 690 pfu/mL was confirmed via agarose plaque assay. Animals were placed back into cages and observed until they had regained reasonable mobility.

Verification of Target Dose and Viremia. Challenge target dose was verified via agarose−based plaque assay. Dilution points were serially diluted 10−fold in EMEM and adsorbed onto Vero E6 cell monolayer in six−well plates. These plates were incubated 1 h at 37 °C−5% CO2 with rocking approximately every 15 min, then immobilized with 2 mL of a 1:1 medium [2× Eagle’s Basal Medium with Earle’s salts (EBME), 10% (vol/vol) FBS, 1% antibiotics] and 1% (vol/vol) (Lonza) mixture. Plates were then incubated for 7 d at 37 °C−5% CO2. After incubation, a neutral red stain medium (2× EBME, 10% FBS, neutral red) was added and plates were incubated with 1% (vol/vol) agarose, and 2 mL was placed into each well. After an additional 24 h of incubation at 37 °C−5% CO2, plates were counted and viremia titers were calculated. Viremia analyses on serum samples and post necropsy−processed tissues were performed using the same method.

Treatment Preparation. Treatments were comprised of a three−mAb mixture. Each mAb was equally represented in the treatment mixture. EU/mL values for each mAb were averaged together and were less than 0.5 EU/mL. Treatment for each group was set at 50 mg/kg and was adjusted via i.v. infusion using 60−mL syringes (BD) and syringe pumps (Lomir Biomedical Inc.). The i.m. and i.p. dosing remained consistent in experimental groups, whereas maximum rate of i.v. infusion was calculated based on endotoxin levels of treatment and animal weight. The infusion rates did not exceed safe volume requirements as determined by the United States Army Medical Research Institute of Infectious Diseases Institutional Animal Care and Use Committee (IACUC) and remained within Food and Drug Administration limits for human use (<5 EU·kg−1·h−1). Animals were periodically monitored during i.v. treatment and for at least 5 min after placement of a new treatment syringe. After the initial triroute treatment, subsequent treatments were given only by i.v. infusion on predetermined days. RHMs in the two pilot studies were treated on days 0 (1 h p.i.), 4, and 8. The third study included RHMs in 24−h (n = 3) and 48−h (n = 3) treatment groups, treated on days 1/2, 5/6, 8, and 10, respectively. The irrelevant mAb control (n = 1) was paired with the 24−h group, and the PBS control (n = 1) was paired with the 48−h group in terms of treatment schedule.

Animal Monitoring and Sample Collection. RHMs were monitored at least once a day for changes in health and diet, as well as notable deviations in behavior. Due to catheterization for the duration of the study, animals were not anesthetized; consequently, weight and temperature data points were not collected. Blood was collected on days 0, 3, 6, 10, 14, 21, and 28 via the external catheter line for complete blood count and chemistry analyses. Blood was also collected on days of euthanasia, time points where animals

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succeeded to challenge (if possible), and at IACUC-approved time points to determine if optional treatments were required.

Complete blood count analysis was performed using the Coulter Act 10 (Beckman Coulter) on samples collected in EDTA plasma vacutette tubes (Greiner Bio-One). Blood was collected in 2-mL serum vacutette tubes (Greiner Bio-One) and allowed to clot for 30 min before use. These samples were spun for 15 min at 500 x g, and the resulting serum was then used in Piccolo 13 discs for chemistry analysis. Additional serum and plasma samples were distributed and frozen at −80 °C for further analysis.

Experiments were conducted under BSL-4 containment conditions; approval for experiments and animal manipulations was given by the USAMRIID IACUC. Animal work was completed by Association for Assessment and Accreditation of Laboratory Animal Care certified staff and under NIH guidelines (41).

Necropsy and Tissue Processing. Necropsies were performed on all RMs. Tissues collected were from liver, kidney, spleen, adrenal, pancreas, and inguinal lymph node.

Tissues were processed using Miltenyi Biotec M tubes and GentleMacs apparatus. All tissues were weighed, and a 10% homogenate (wt/vol) was created in appropriate volumes of EEMEM. The homogenates were stored at −80 °C for analysis as needed.

ELISA. ELISAs were performed using a recombinant EBOV glycoprotein (National Cancer Institute), and plasma samples serially diluted at half-log increments. Goat anti-human IgG (Heavy + Light) (KPL) and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Millipore) were used as secondary and substrate, respectively. Assays were read at 405 nm absorbance (SpectraMax M5; Molecular Devices).