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# Novel neutralizing monoclonal antibodies protect rodents against lethal filovirus challenges



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## ABSTRACT

Filoviruses are the causative agents of lethal hemorrhagic fever in human and non-human primates (NHP). The family of *Filoviridae* is composed of three genera, *Ebolavirus*, *Marburgvirus* and *Cuevavirus*. There are currently no approved vaccines or antiviral therapeutics for the treatment of filovirus infections in humans. Passive transfer of neutralizing antibodies targeting the Ebola virus (EBOV) glycoprotein (GP) has proven effective in protecting mice, guinea pigs and NHP from lethal challenges with EBOV. In this study, we generated two neutralizing monoclonal antibodies (MAbs), termed S9 and M4 that recognize the GP of EBOV or multiple strains of Marburg virus (MARV), respectively. We characterized the putative binding site of S9 as a linear epitope on the glycan cap of the GP<sub>1</sub> subunit of the EBOV-GP. The M4 antibody recognizes an unknown conformational epitope on MARV-GP. Additionally, we demonstrated the post-exposure protection potential of these antibodies in both the mouse and guinea pig models of filovirus infection. These data indicate that MAbs S9 and M4 would be good candidates for inclusion in an antibody cocktail for the treatment of filovirus infections.

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## 1. Introduction

The family of *Filoviridae* is composed of three genera, *Ebolavirus*, *Marburgvirus* and *Cuevavirus*. The *Ebolavirus* genus can be subdivided into 5 distinct species: Zaire (EBOV), Sudan (SUDV), Tai Forest (TAFV), Reston (RESTV) and Bundibugyo (BDBV) viruses. The *Marburgvirus* genus comprises of a single species, *Marburg marburgvirus*, of which several strains have been reported, including its prototype virus Marburg virus (MARV) [1]. Infection with EBOV and MARV results in a rapidly fatal hemorrhagic fever in humans with reported case fatality rates of up to 90% [2,3].

Currently, there is no approved treatment for EBOV or MARV hemorrhagic fever beyond palliative care [4].

Attachment and entry of filoviruses into target cells is mediated by the viral glycoprotein (GP), which is the sole viral protein exposed on the virion surface [5]. The GP is post-translationally cleaved into two subunits, GP<sub>1</sub> and GP<sub>2</sub>, both of which form a trimer of heterodimers. The filovirus GP is composed of a heavily glycosylated mucin domain and glycan cap region, which form a deep, heavily glycosylated, chalice-like structure that encloses the putative receptor-binding domain [6].

To date most research evaluating the potential of passive antibody transfer as a therapy for filovirus infections has focused on EBOV infections. Neutralizing antibodies that target the EBOV-GP have proven effective in protecting mice, guinea pigs and more recently non-human primates (NHP) from lethal EBOV challenge [7–12], while neutralizing monoclonal antibodies (MAbs) against MARV GP were shown to confer only partial protection in guinea pigs [13].

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In this study, we characterized 2 novel neutralizing MAbs against EBOV and MARV, and show that both MAbs protect against lethal EBOV or MARV challenge in mice and/or guinea pigs.

## 2. Materials and methods

### 2.1. Ethics statement

Approval for animal experiments was obtained from the Institutional Animal Care and Use Committees at Rocky Mountain Laboratories, DIR, NIAID, NIH and the University of Texas Medical Branch. Animal work was performed by certified staff in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facility. Animal housing, care and experimental protocols were in accordance with NIH guidelines.

### 2.2. Viruses and cells

Wildtype (WT) EBOV (strain Mayinga) was kindly provided by the Special Pathogens Branch of the Centers for Disease Control and Prevention, Atlanta, Georgia, United States, mouse adapted (MA)-EBOV (strain Mayinga) and guinea pig adapted (GPA-) EBOV (strain Mayinga) were kindly provided by United States Army Medical Research Institute of Infectious Diseases, Frederick, MD, US and WT MARV (strain Angola) and GPA-MARV (strain Angola) were kindly provided by Public Health Agency of Canada, Winnipeg, Canada. Generation of recombinant VSV expressing EBOV-GP and MARV-GP has been described previously [14]. All viruses were grown in Vero E6 cells in Dulbeccos modified eagles medium (DMEM) supplemented with 2% Fetal Bovine Serum (FBS) and antibiotics. Hybridoma cells were grown in DMEM supplemented with 20% FBS, antibiotics, L-glutamine, and 10 mM HEPES at 37 °C and 5% CO<sub>2</sub>. All work was performed in a class II biological safety cabinet. All work with live EBOV or MARV was performed under biosafety level 4 conditions at the Integrated Research Facility, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana and the Galveston National Laboratory, University of Texas Medical Branch, Galveston, Texas.

### 2.3. Generation of antibody producing hybridomas

The neutralizing MAbs were generated through a project originally designed to induce cross-reactive MAbs. Therefore, groups of BALB/c mice were vaccinated with  $3 \times 10^5$  plaque forming units (PFU) rVSV/EBOV-GP. Three weeks after vaccination, mice were boosted with  $3 \times 10^5$  rVSV/SUDV-GP or rVSV/MARV-GP to induce a memory response against cross-reactive epitopes. Three days after boost the mice were sacrificed, spleens harvested, and hybridomas generated as described previously [15]. Briefly, mouse plasma cells were fused with SP2/0-Ag14 myeloma cells (ATCC). Hybridomas were selected using HAT/HT selection medium. Monoclonal hybridomas were isolated by two rounds of limiting dilutions in 96 well flat bottom tissue culture plates. Isolated hybridomas were screened for secreted antibodies using a soluble transmembrane deleted trimeric glycoprotein of EBOV, SUDV, RESTV, TAFV, BDBV and MARV via indirect ELISA as previously described [16]. Plasmids for soluble GP were kindly provided by Dr. Ayato Takada.

### 2.4. Monoclonal antibody purification

Antibody was purified from hybridoma cell culture supernatant by concentration using Amicon plus-70 30,000 MWCO centrifugal concentrators followed by protein A/G purification columns

(Thermo Scientific) according to manufacturer's guidelines. Antibody was buffer exchanged into PBS using an Amicon 15 ml centrifugal spin concentrator. Purified antibody concentrations were determined by Bradford Protein Assay Reagent (Thermo Scientific).

### 2.5. Plaque reduction assay

MAbs were serially diluted twofold in DMEM and added to 100 plaque forming units (PFU) of rVSV/EBOV-GP, rVSV/MARV-GP, WT EBOV or WT MARV and incubated for 1 h at 37 °C. The MAb/virus mix was used to infect monolayers of VeroE6 cells for 1 h at 37 °C. Following adsorption, the inoculum was removed and the cells were overlaid with 0.8% agarose/MEM/10% FBS. Cells were incubated for two days with rVSV or 5–7 days with WT MARV or EBOV, respectively, after which cells were stained with crystal violet and plaques counted. Neutralization was calculated as the % reduction in plaques as compared to untreated virus.

### 2.6. Western blotting

Purified soluble EBOV- and MARV-GP samples were analyzed by SDS-PAGE using 10% acrylamide gel and transferred onto Hybond-P PVDF Membrane (GE Healthcare) utilizing a Bio-Rad, Trans-Blot SD Semi-Dry Transfer Cell following the manufacturer's instruction. The membrane was blocked with 5% non-fat milk PBS/0.1% tween overnight at 4 °C and incubated with 2 µg/ml S9 or M4 MAb diluted in 5% non-fat milk/PBS-T for 1 h. The membrane was then washed three times in PBS-T and incubated with anti-mouse HRP antibody (1:25000; Jackson Immunoresearch), for 1 h at room temperature. Following a final washing step the binding of antibody to the membrane was detected by ECL Plus Western Blotting Detection Reagent and Hyperfilm ECL (GE Healthcare, Amersham).

### 2.7. Escape mutants

Escape mutants were generated by incubating  $10^5$  TCID<sub>50</sub> rVSV/EBOV-GP or rVSV/MARV-GP with a sub-neutralizing amount (5 µg/ml) of MAb S9 or M4 for 1 h at 37 °C. The mixture was then inoculated onto a monolayer of Vero E6 cells for 1 h at 37 °C. Cultures were checked for CPE and viruses that escaped neutralization were then passaged two additional times in the presence of MAb until resistant viruses were obtained. Neutralization escape mutants were plaque purified in the presence of MAb, and RNA from six individual plaques was isolated using TRIzol Reagent (Life Technologies) according to the manufacturer's guidelines. The GP gene was amplified from the viral RNA using primers flanking this gene in the rVSV backbone by RT-PCR and sequenced.

### 2.8. Peptide epitopes

Overlapping synthetic pin peptides for the complete amino acid sequence of EBOV-GP (GenBank accession number NP\_066246.1) were synthesized on pins as 15-mer peptides with a 10-mer overlap of each peptide (PepScan Presto). The pins were used to determine the linear binding epitope of antibodies. Briefly, pins were blocked with 1% Skim milk (Bio-Rad) + 1% Tween-20 (Fisher) in 0.01 M PBS in a Nunc round-bottom 96 well plate for 2 h at room temperature then washed in 0.9% w/v NaCl and 0.05% v/v Tween-20 in PBS (pH 7.2). Concentrated hybridoma supernatant for mAbs were diluted 1:1 in 0.1% Skim milk + 0.1% Tween-20 in 0.01 M PBS and added to a new flexible Falcon 96 well plate and the pins were incubated overnight at 4 °C in the antibody solution. Next, the pins were washed as described above and an appropriate dilution of a goat anti-mouse IgG-HRP secondary antibody (Southern Biotech) in 0.1% Skim milk + 0.1% Tween-20 was incubated with the pins

for 1 h at room temperature. Pins were washed as before and then developed in ABTS solution (Roche) and incubated at room temperature while color development was monitored. The plate was read at 30 and 60 min at 405 nm by use of a SpectroMax250 Spectrometer (Molecular Devices), and recorded by SoftMax Pro 4.6 Software (Molecular Devices). Pins were cleaned for subsequent analysis according to commercial instructions.

### 2.9. Structural mapping of epitope

The peptides identified using the pepscan method were mapped onto the crystal structure of EBOV-GP<sub>1</sub> (PDB id: 3CSY) [6]. Since the coordinates from K190 to Y213 (segment1) and N278 to R299 (segment2) are missing in the crystal structure of EBOV-GP<sub>1</sub>, these segments were modeled onto the existing EBOV-GP<sub>1</sub> crystal structure based on homology with other proteins. Briefly, the sequence of EBOV (GenBank id: NP\_066246.1) was submitted to a fold recognition server to find a suitable template structure for the missing segments. In the absence of a good template structure, segment1 was modeled as a random loop structure, while segment2 was modeled based on homology with PDB id: 3DSL [17]. Finally, the MPACK program was used to build the complete model structure of EBOV-GP<sub>1</sub> by combining the two template structures (PDB id: 3CSY and 3DSL), similar to previous 3D modeling efforts in our group [18,19]. The model structure of EBOV-GP<sub>1</sub> was energy minimized using Fantom program [20]. The overall root-mean-square deviation (r.m.s.d.) between model and crystal structure of EBOV-GP<sub>1</sub> protein was found to be 0.32 Å. Figures were generated using the PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.

### 2.10. Animal experiments

Groups of 6 female BALB/c mice (6 weeks of age) as well as 4 female Hartley guinea pigs (about 250 g) were obtained from Charles River Laboratories and housed in HEPA-filtered micro-isolator cage systems. Mice were infected with 500× lethal dose 50% (LD<sub>50</sub>) of MA-EBOV by the intraperitoneal (IP) route. On days -1, 0, 1, 2, 3 post-infection, groups of 6 mice were given a single injection of 250 µg/mouse purified MAb S9 via the IP route. Mice were weighed daily and monitored for clinical signs of disease up to 34 days after challenge. All surviving animals were euthanized and bled on day 34 post challenge. Groups of 4 guinea pigs were infected with 500× LD<sub>50</sub> of GPA-EBOV (10 focus-forming units) or GPA-MARV (1000 Tissue Culture Infectious Dose 50%) via the IP route. On days 0, 1, 2 or 3 animals received a single dose of MAb S9 or M4, respectively, (~5 mg per animal) in 5 ml PBS via the IP route. Animals were monitored for development of clinical signs and weight loss daily until day 14. Surviving animals were euthanized at day 21.

## 3. Results

### 3.1. Identification of neutralizing antibodies

Mice were primed with rVSV/EBOV-GP, followed by a boost with rVSV/SUDV-GP or rVSV/MARV-GP. Two potent neutralizing MAbs were identified during initial screening, one against EBOV (S9) and one against MARV (M4). To determine the potency of neutralization, as well as the cross-neutralizing potential of the two monoclonal antibodies, a plaque reduction assay was performed. MAb S9 neutralized rVSV/EBOV-GP (Fig. 1A), but no other rVSVs pseudotyped with GPs derived from distinct *Ebolavirus* species (SUDV, RESTV, TAFV, BDBV; data not shown). MAb S9 showed 50% neutralization at 1 µg/ml and 90% neutralization at 4 µg/ml.

MAb M4 not only potently neutralized rVSV/MARV-GP (Angola strain), but also rVSVs pseudotyped with GPs derived from distinct MARV strains (Musoke, Ozolin and Ravn) (Fig. 1B). M4 showed 50% neutralization at 0.1 µg/ml and 90% neutralization at 0.6 µg/ml.

### 3.2. Neutralizing epitope characterization

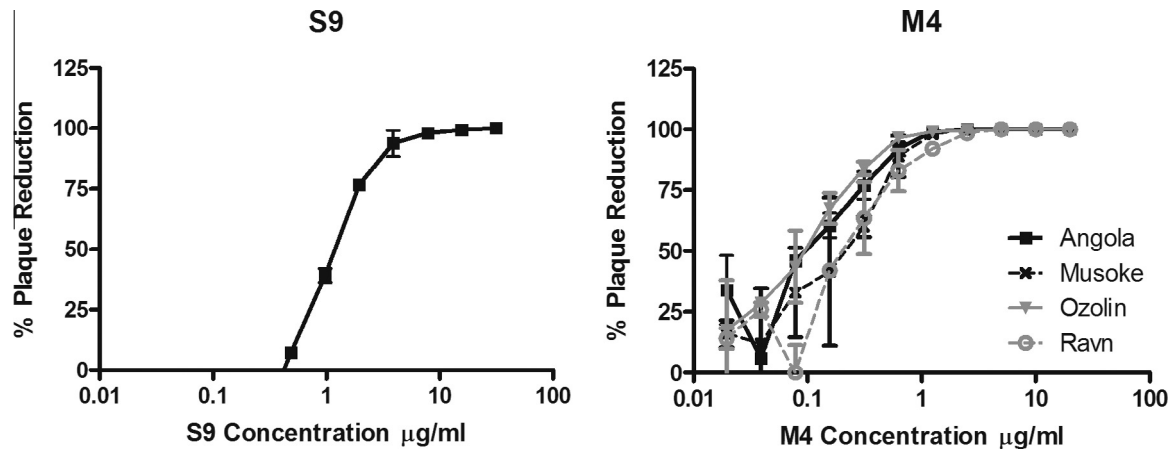
To evaluate if MAbs S9 and M4 bind conformational or linear epitopes, binding to EBOV- or MARV-GP was determined by Western blot under reducing conditions eliminating conformational epitopes. The western blot analysis revealed a band at 140 kDa, the expected size of the GP<sub>1</sub> subunit [21], for S9 but not for M4 (Fig. 2), while neither antibody reacted with GP<sub>2</sub> (26 kDa). These data suggest that MAb S9 recognizes a linear epitope on EBOV GP<sub>1</sub>, whereas MAb M4 most likely recognizes a conformational epitope on the MARV GP. To further characterize the linear epitope of S9, Pepscan's CLIPS epitope mapping technology was used. Pepscan's CLIPS Epitope Mapping technology utilizes synthesized, overlapping, structurally constrained peptides that cover the entire sequence of the EBOV GP. MAb S9 reacted strongest with the EBOV-GP peptide sequence TKKLNLRKIRSEELSC, at an Optical Density of 0.439, which corresponds to positions 293–307 on the GP<sub>1</sub> subunit of the glycoprotein. Interestingly, alignment of this epitope domain in EBOV with those from RESTV, BDBV, TAFV and SUDV shows up to 60% divergence between EBOV and RESTV (Table 1).

To further characterize the binding site of MAbs S9 and M4, rVSV/EBOV-GP and rVSV/MARV-GP were grown in the presence of MAb M4 or S9 to select for antigenic variants that escape MAb mediated neutralization. No escape mutant could be generated using the MAb M4 after 3 independent attempts. In contrast, six plaque purified escape variants were sequenced for S9 and all had a single mutation of G to A at nucleotide position 811 of the EBOV-GP resulting in an amino acid substitution from glycine (G) to arginine (R) at position 271 located in the glycan cap region of GP<sub>1</sub> (Fig. 3A and B). This neutralization escape mutation at G271R is in close proximity to the N-terminus of the EBOV-GP peptide sequence on the EBOV-GP crystal structure.

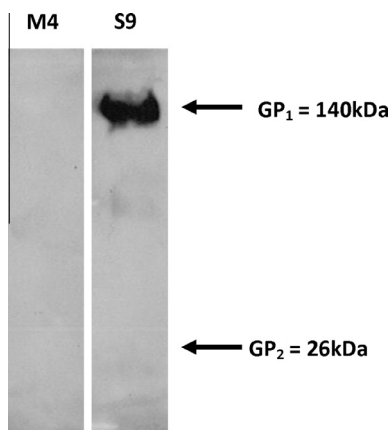
### 3.3. Neutralizing antibody protects mice and guinea pigs from a lethal filovirus challenge

The therapeutic potential of MAb S9 was first evaluated in a mouse model. All mice treated with a single dose of MAb S9 starting on days -1, 0, 1 or 2 survived a lethal challenge with MA-EBOV (Fig. 4). However, mice treated with MAb S9 starting 3 days post-exposure did not show significantly prolonged time to death and succumbed to infection 5–8 days post infection similar to Mock (PBS)-treated animals (Fig. 4).

To further evaluate the efficacy of MAb S9 and to determine the efficacy of MAb M4 we tested the therapeutic efficacy of these antibodies in a lethal guinea pig model for both EBOV and MARV. A single dose of MAb S9 conferred complete protection to all EBOV-infected guinea pigs treated directly following infection (Fig. 5A). Additionally, MAb S9 displayed efficacy in protecting half of the guinea pigs from a lethal GPA-EBOV challenge when administered up to one day post-infection and increased the time to death up to 3 days (Fig. 5A). A single dose treatment with MAb M4 protected guinea pigs challenged with a lethal dose of GPA-MARV when administered up to 3 days after infection. All (100%) challenged animals were protected when treated at the time of infection; whereas 50–75% of the animals were protected when MAb M4 was administered up to 3 days post infection (Fig. 5B). No significant difference in protective efficacy was observed between animals treated 1, 2 or 3 days post infection.



**Fig. 1.** Neutralization of EBOV and MARV strains by S9 and M4 monoclonal antibodies by plaque reduction assay. Shown are the percentages of plaque reduction of EBOV (Mayinga strain) and MARV (Angolan, Musoke, Ozolin and Ravn strains) by S9 and M4, respectively, compared to untreated controls. Error bars represent standard deviations from triplicates.



**Fig. 2.** Reactivity of M4 and S9 with linear epitopes on the GP. Shown is the Western blot analysis of M4 and S9 MAb binding to recombinant soluble forms of MARV- or EBOV-GP. Expected sizes of GP<sub>1</sub> and GP<sub>2</sub> are marked at 140 and 26 kDa, respectively.

#### 4. Discussion

Promising prophylactic treatments using antibodies against EBOV have been explored using small rodent models, and more recently with NHP challenged with EBOV. The best characterized neutralizing MAb, KZ52, was derived from a human survivor of the 1995 Kikwit EBOV outbreak [8]. The crystal structure of KZ52 was solved in complex with the EBOV-GP [6]. KZ52 binds to unglycosylated regions on both GP<sub>1</sub> and GP<sub>2</sub> at the base of the chalice-like structure of the GP. Clamping of GP<sub>1</sub> and GP<sub>2</sub> subunits by KZ52 neutralizes the virion by inhibiting essential conformational changes of the glycoprotein that facilitate fusion of the viral membrane with the host cell membrane [22]. This antibody showed efficacy in pre- and postexposure prophylaxis in a guinea pig

model, but failed to protect against lethal challenge in a NHP model [8,23]. Mouse-derived neutralizing MAb 133/3.16 binds a linear epitope that likely overlaps with KZ52 located at the base of the EBOV GP chalice [24]. MAb 226/8.1 binds a conformational epitope comprising GP residues 134, 194, and 199 in the vicinity of the cathepsin cleavage site [24,25]. The recently described MB-003 MAb cocktail contains 3 MAbs that are believed to target various regions within the mucin domain [26]. Finally another cocktail of 3 MAbs designated ZMAb is believed to target the base, the GP2 and the mucin domain [27]. These neutralizing MAbs and others have been shown to protect mice, guinea pigs and NHP from a lethal EBOV challenge [9–12].

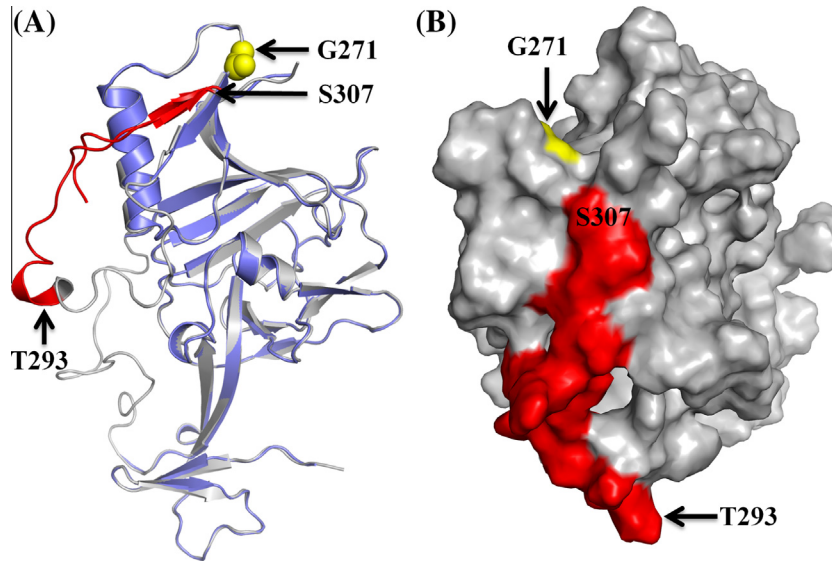
In this study, using multiple complimentary methods, we generated and characterized the neutralizing MAbs M4 and S9, which bind to the GP of MARV and EBOV, respectively. In order to identify the neutralizing epitopes recognized by MAbs, we and others have previously employed neutralization escape mutant analysis [25,28,29]. While we were unable to recover infectious MARV during passage in the presence of MAb M4, we were successful in identifying a G271R change in the EBOV-GP as a correlate of neutralization escape to MAb S9. Further analysis through western blot and pepscan epitope mapping suggests that the epitope recognized by MAb S9 is linear and comprises of residues at positions 293–307.

Together these data suggest that the G271R change caused an opposing interaction between the positively charged R and the neighboring positively charged K, thus likely rearranging the conformation on the surface of the GP<sub>1</sub> portion of the epitope causing disruption of the MAb S9 binding site on GP<sub>1</sub>. The mechanism of neutralization of MAb S9 is most likely due to direct blocking of the virus–receptor interaction. Since recent studies have shown that neutralizing antibodies can protect against lethal EBOV challenge in a variety of species including guinea pigs and NHP [8–12,30]. We used a well established mouse model of lethal EBOV infection [30] to show that MAb S9 protects mice completely from

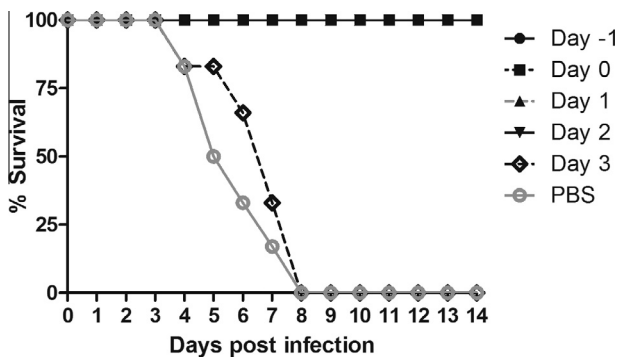
**Table 1**  
Ebola virus amino acid sequence variation in S9 epitope. Shown is the amino acid sequence alignment of the S9 epitope for Zaire (EBOV), Reston (RESTV), Tai Forest (TAFV), Sudan (SUDV) and Bundibugyo (BDBV) viruses. \* = identical amino acid.

Virus	GenBank	Epitope														
EBOV	U23187	T	K	K	N	L	T	R	K	I	R	S	E	E	L	S
RESTV	U23152	*	*	*	*	F	S	Q	Q	L	H	G	*	N	*	H
TAFVV	U28006	N	*	*	*	F	*	K	T	L	S	*	*	*	*	*
SUDV	U28134	*	*	*	*	*	S	E	Q	L	*	G	*	*	*	*
BDBV	FJ217161	N	*	*	*	F	*	K	T	L	S	*	*	*	*	*





**Fig. 3.** Location of the S9 epitope on the EBOV-GP structural model. Shown is the structural mapping of the S9 epitope on a cartoon representation (A) and surface view (B) based on escape mutation (residue 271 in yellow) and Pepsan analysis (residues 293–307 in red).



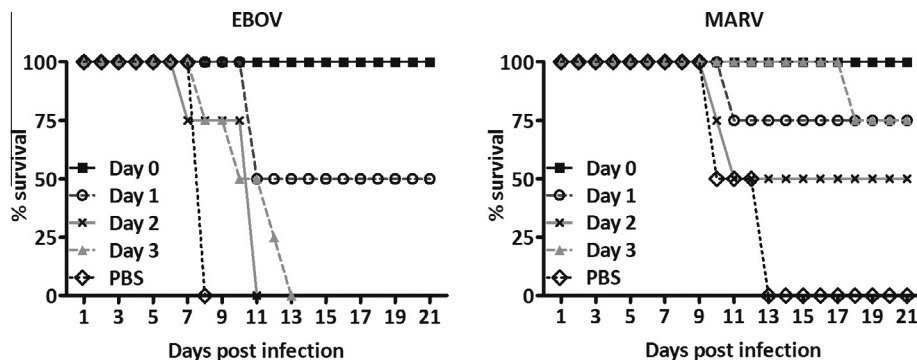
**Fig. 4.** Prophylactic treatment of lethal EBOV infection in mice with S9. Shown is the percentage of survivors of mouse-adapted EBOV infected mice treated with a single dose of S9 (250 µg) at various days post challenge (n = 6 per treatment).

lethal EBOV challenge when initiating treatment up to 2 days post infection demonstrating both a prophylactic and therapeutic use of MAb S9. Additionally, MAb S9 completely protected guinea pigs when treated the day of infection, and provided partial protection up to 2 days post infection. Interestingly, protective efficacy up to 3 days post infection was observed by increasing the time to death. These data suggest that post-exposure treatment with MAb S9

would increase the therapeutic window and could be combined with other treatments such as RNA interference [31] or interferon treatment [32]. Since MAb S9 is a neutralizing antibody with a binding site on the glycan cap that is distinct from most other previously published neutralizing antibodies that primarily bind the base subdomain or mucin domain [6,25,27], we believe MAb S9 would also be a good candidate for inclusion in a neutralizing MAb cocktail that could provide broad prophylactic and therapeutic protection against EBOV infections in NHP.

To date there has been limited information on neutralizing MAbs directed against different MARV strains [13]. Here we report a potent neutralizing MAb M4 that is likely directed against a conformational epitope on MARV-GP. The fact that no neutralization escape mutant could be generated, suggests that MAb M4 is targeting an area on the MARV GP that is crucial for virus entry and/or viability. This antibody is capable of potently cross neutralizing different MARV isolates at nanomolar concentrations. Additionally, this antibody protects guinea pigs from a lethal challenge of MARV with efficacy up to 3 days post-infection. Given the recent success of antibody treatment against EBOV, the M4 neutralizing antibody will be a promising candidate for passive prophylactic therapy against MARV infections in NHPs.

In conclusion, we have identified two novel neutralizing MAbs against EBOV and MARV that provide prophylactic and therapeutic



**Fig. 5.** Prophylactic treatment of lethal filovirus infection in guinea pigs with S9 and M4. Shown is the percentage of survival in groups of guinea pigs (n = 4) infected with guinea pig-adapted EBOV (A) or MARV (B) and treated with a single dose of S9 or M4 (5 mg) at various days post challenge.

protection against lethal infection in animals. These MABs are attractive candidates for inclusion in MAB cocktails to provide broad protection against multiple EBOV species and MARV strains.

### Conflict of interest

The authors declare no conflict of interest.

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