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Mechanistic and Fc requirements for inhibition of Sudan virus entry and *in vivo* protection by a synthetic antibody★

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Abstract

The Sudan virus (SUDV), an ebolavirus, causes severe hemorrhagic fever with human case fatality rates of ~50%. Previous work from our lab demonstrated the synthetic antibody F4 potently inhibits viral entry and protects against lethal virus challenge in mice [Chen et al., ACS Chem. Biol., 2014, 9, 2263–2273]. Here, we explore mechanistic requirements as well as contribution of the Fc region and function on neutralization and *in vivo* protection. Live cell imaging demonstrates that the antibody colocalizes with vesicular stomatitis virus particles containing the Sudan virus glycoprotein (VSV-GP_{SUDV}) and that the antibody is rapidly degraded within cellular endosomes. A viral escape mutant contained substitutions on the N-heptad repeat (NHR) segment of GP2, the fusion subunit. Truncation studies indicated that the size of the Fc impacts virus neutralization potential. Finally, we examined the protective efficacy of Fc-null mutants in mice, and found that Fc function was not required for high levels of protection. Altogether, these results indicate that neutralization of SUDV GP-mediated cell entry likely involves blockade of viral membrane fusion within endosomes, and that inhibition of viral entry is the likely mechanism of *in vivo* protection.

Keywords

Sudan virus; Ebola virus; Neutralizing antibodies; Synthetic antibodies

★During publication of this article, Wec et al. (Cell, 2017, 169, 878) and Zhao et al. (Cell, 2017, 169, 891) described cross-protective antibodies from humans and macaques with activity against SUDV in mice.

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Declaration of interest

The United States Army Medical Research Institute of Infectious Disease (USAMRIID) has filed a patent application on the murine mAb 16F6 (WO2011071574 A2) entitled “Monoclonal Antibodies against Glycoprotein of Ebola Sudan Boniface Virus” with J.M.D. as a coinventor. The Albert Einstein College of Medicine has filed a patent application on F4 (U.S. Provisional Patent Application No. 61/830,325 and U.S. Utility Application No. 14/291,608) entitled “Therapy for Filovirus Infection” with S. E. Z., J. M. D., and J. R. L. as coinventors.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.imlet.2017.09.002>.

1. Introduction

Members of the *ebolavirus* family cause severe hemorrhagic fever with a high percentage of fatal cases. Five different ebolaviruses have been isolated: Ebola virus (Zaire, EBOV), Bundibugyo virus (BDBV), Tai Forest virus (TAFV), Reston virus (RESTV), and Sudan virus (SUDV). Among these, EBOV and SUDV are responsible for most of the ebolavirus-related deaths [1]. The 2014–2016 EBOV epidemic in West Africa far exceeded the scale of any previous ebolavirus outbreak, with over 28,000 suspected cases of infection [2]. Prior to 2014, the largest ebolavirus outbreak was caused by SUDV in 2000, where at least 425 individuals were infected and the mortality rate was ~50% [3]. Monoclonal antibodies (mAbs) represent a promising therapeutic platform for the treatment of ebolavirus infections. For example, the mAb cocktail ZMapp™ (Mapp Biopharmaceutical) has been shown to reverse the course of advanced Ebola virus disease (EVD) in non-human primates and recently completed clinical efficacy studies (PREVAIL II) [4]. However, although much effort has been made to isolate mAbs against EBOV, only a few mAbs have been shown to protect *in vivo* from lethal SUDV challenge [5–10]. The glycoproteins for EBOV and SUDV are ~45% divergent and thus mAbs that can cross-neutralize these viruses are rare [7,8,10]. We previously employed antibody engineering approaches to develop cross-protective ebolavirus antibodies [6,11].

The filovirus glycoprotein GP is the primary target of neutralizing antibodies [12–14]. The mature spike of the glycoprotein is a trimer comprised of three disulfide-linked GP1–GP2 heterodimers that are generated by furin cleavage during virus assembly. The prefusion GP1–GP2 spike displays a chalice-and-bowl morphology. Three GP2 subunits form the chalice, while the bowl is represented by three GP1 subunits [12]. The head of GP1 contains a putative receptor-binding site (RBS). The glycan cap and the mucin-like domain of the glycoprotein are extensively glycosylated and, in the prefusion form, likely sequester the critical RBS from the adaptive immune response [11,15,16]. Virus entry into cells is initiated by the interaction of GP1 with multiple cell-surface molecules and proceeds *via* a macropinocytosis-like mechanism [17]. During the endolysosomal transport of the virus, major segments of GP1 (the glycan cap and mucin-like domain) are removed by host endosomal cysteine proteases (Cathepsin L and Cathepsin B for EBOV). Next, the RBS engages the critical host receptor, Niemann Pick C1 (NPC1) *via* its luminal C-loop [12,18]. Subsequent events lead ultimately to conformational changes in the GP2 subunit that promote viral membrane fusion and delivery of the viral contents into the cytosol [19–21].

A few studies to date have examined the intracellular fate of neutralizing ebolavirus mAbs, however these have all focused on EBOV. EBOV mAbs bind a number of epitopes on the viral glycoprotein and can potentially mediate different neutralizing effector functions [13]. The most potent antibodies bind epitopes at the GP1–GP2 interface, the glycan cap or the stem (C-heptad repeat region, CHR) of the glycoprotein [12,13]. Several studies suggest that protective antibodies directly inhibit the membrane fusion event between host and virus by either impeding necessary glycoprotein conformational changes or protection of the glycoprotein against the required proteolytic disassembly that reveals the RBS [12,22,23]. A number of neutralizing mAbs bind at the GP1–GP2 interface, and one such EBOV-specific mAb, KZ52, seem to be able to protect GP from Cathepsin L-dependent activation, and thus

may potentially block infection by preventing release of the GP2 fusion loop into the host endosomal membrane [23,24]. Here, we investigate the role of Fc for SUDV mAb F4 that was previously shown to provide potent post-exposure protection from viral challenge in mice [5]. The design of F4 is based on the humanized framework regions of Herceptin® and the complementarity determining regions (CDRs) of murine SUDV antibody 16F6 [5]. A synthetic antibody phage display approach was employed to impart 16F6-like specificity onto the humanized Herceptin® scaffold. F4 exhibits strong neutralization potential against SUDV, as well as potent post-exposure protective efficacy in mouse studies [5]. However, a detailed study on the mechanism of action is lacking so far, though would contribute significantly to knowledge about neutralizing SUDV antibodies and potentially inform engineering efforts to improve activity.

2. Material and methods

2.1. Expression and purification of IgGs and antibody fragments

HEK293FS cells were seeded at 500 000 cells/ml a day prior to expression. For expression of full length IgG1s, vectors for the heavy and light chain were transfected into HEK293FS cells (Invitrogen) by using linear polyethylenimine (Polysciences). Six days posttransfection, the cells were centrifuged and the supernatants were applied to a protein-A affinity beads (~2 mL packed beads per 600 mL culture) (Pierce). Immunoglobulins were washed with Protein A gentle binding buffer and then eluted with gentle elution buffer (both ThermoFisher). The eluent was buffer exchanged by using PD-10 columns (GE healthcare) and concentrated by using a 30k Amicon centrifuge tube. F4-CH2 was generated by introducing a stop codon after the constant CH2 domain and purified as described above. F4 (Fab)₂ and F4-Fabs were generated by the introduction of a 6× HIS tag and a stop codon after the hinge region and the CH1 region, respectively. These truncated antibody fragment DNA segments were cloned into an IPTG-inducible expression vector pFabHis-MBP [30] and transformed into BL21 star (DE3) (Life technologies). After growing the preculture in 2×YT media to an OD of 0.4, 1 mM IPTG was added and periplasmic expression was induced for 4 h. Bacteria were harvested, lysed with bugbuster (Millipore) and supernatant was loaded onto Ni-NTA beads for 2 h. F4-(Fab)₂ and F4-Fab molecules were loaded onto a column and eluted with 500 mM imidazole after washing. Subsequently, antibody fragments were washed several times with PBS and concentrated in a 10k Amicon centrifuge tube. Concentrations were determined by nanodrop and SDS-PAGE was performed to analyze the size of the molecules.

2.2. Confocal live cell imaging

Live cell imaging was performed on a confocal spinning disc microscope (PerkinElmer) with an inverted Nikon TE2000-S. Vero cells were grown inside iBidi treat 8-wells and incubated with F4-neoVSV-GP_{SUDV} complex. Prior to this, F4 was directly labeled with the APEX antibody labeling kit (Alexa-Fluor 647) and tested for binding to neoGP_{SUDV}. Recording of virus internalization and intracellular trafficking was started 10 min after exposure. Images of the two channels were recorded every 30 s.

2.3. Inhibition experiments

Experiments on the rescue of antibody degradation were performed by using the Cathepsin inhibitor E64d [25]. To inhibit the endosomal proteases, Vero cells were pretreated with 300 μ M of E64d for 60 min. neoVSV-GP_{SUDV} particles complexed with 40 μ g ml⁻¹ F4 and added to the cells. 60 min later, cells were washed twice with PBS, fixed with 2% paraformaldehyde and then processed for immunofluorescence stainings and microscopy. Antibodies were detected with a secondary anti-IgG1-AF555 antibody (1:200). Colocalization was measured by correlation of the virus channel (green) with the antibody channel (red) using the colocalization plugin of ImageJ.

2.4. Generation of rVSV-GP_{SUDV} escape mutants

Escape mutants were generated as previously described [26]. Recombinant rVSV-GP_{SUDV} was pre-incubated at a concentration of 2.5×10^3 PFU/ml with 10 nM of F4 or 16F6 for 60 min at 37 °C. Vero cells in a 6-well plate were infected for 2 h and then cultured in DMEM (+ 2% FBS) until almost 90% of the cells showed cytopathic effects (CPE). Infection and morphology was checked daily by fluorescence microscopy. Subsequently, supernatants were harvested and incubated with 50 nM of F4 and 16F6 until CPE were detectable. Supernatants were again collected and incubated with 200 nM of F4 and 16F6 until 90% of the cells were infected. The supernatants from the third round were used for RNA isolation using the Quick-RNA MiniPrep kit from Zymo Research. The region of the VSV-genome containing the SUDV GP was amplified using the Qiagen One-Step RT-PCR kit and the primers 5'-AAGGTGCCGCTATGTTCA, 5'-GAACATACTCAGATGTGG, 5'-AGCTTCCAGCTTCTGAACAATCCC, 5'-ATGCTGCTGGGATTGCCTGGA and 5'-CGACCGGGCCACCAGGAAGT. Sequencing of the cDNA was performed by using the same primers. Escape mutants were identified by sequence alignments of the published GP_{SUDV} sequence.

2.5. Neutralization experiments

Neutralization assays were performed using vesicular stomatitis virus bearing the GP from SUDV instead of its native G glycoprotein (VSV-GP_{SUDV}). The viral genome encodes an enhanced green fluorescent protein (eGFP), and infection scored by counting fluorescent cells after infection. The protocol for VSV-GP_{SUDV} production has been described elsewhere [18]. Experiments were performed in 96-well tissue grade cell culture plates (Corning). At the time point of infection, Vero cells were at 90% confluency. VSV-GP_{SUDV} was pre-incubated with the indicated concentrations of antibody or antibody fragments for 15 min. The antibody-virus mixtures were then added in triplicates to the Vero cells. Infection was carried out overnight. At the next day, cells were fixed, stained with DAPI and forwarded to automatic infection counting using the CellInsight CX5 High Content Screening (HCS) platform (ThermoFisher). The percentage of infected cells (green) were calculated in percentage to the DAPI positive cells.

2.6. ELISA

GP_{SUDV} was directly immobilized onto 96-well Maxisorp plates (GP_{SUDV} = 0.5 μ g/well) by incubating in phosphate-buffered saline (PBS) pH 8.0 for 14–16 h at 4 °C. PBS, pH 7.4,

containing 5% non-fat dry milk (NFDM) was used to block the wells after target immobilization (incubation for 60–90 min at RT). Negative control plates were coated with 5% NFDM only. IgGs were diluted into PBS containing 0.1% (w/v) Tween 20 (PBST), applied to the wells, and incubated at RT for 1 h. The plates were washed with PBST and incubated for 45–60 min with Protein A/horse radish peroxidase (HRP) antibody conjugate (1:1000 dilution in PBST). The wells were washed 4–6 times with PBST and developed as by the addition of using 3, 3', 5, 5'-tetramethylbenzidine (TMB; Sigma-Aldrich, St. Louis, MO), and quenched with 0.5 M sulfuric acid. The absorbance at 450 nm was determined. The data were fit to standard four-parameter logistic equations using Graphpad Prism (GraphPad Software, La Jolla, CA). The half-maximal binding titers (EC_{50}) were obtained from the inflection point in the curves.

2.7. Mouse protection studies

Animal experiments were performed as previously described [5]. Male and female Type 1 IFN α/β receptor knockout mice (IFNAR^{-/-}) purchased from Jackson Laboratory (4 weeks of age) were utilized in these experiments. Mice were intraperitoneally (IP) challenged with a target dose of 1000 plaque forming units (pfu) of wild-type SUDV. Afterwards, animals were IP-treated with 500 μ g of indicated mAb at day + 1 and + 3 postinfection. Surviving mice were rechallenged IP with target dose 1000 pfu of wild-type SUDV with no treatment provided. Following all challenges mice were monitored daily for morbidity and mortality.

2.8. Biolayer interferometry

Affinity constants were determined by biolayer interferometry using the OctetRed (ForteBio). F4-IgG1 was loaded at a concentration of 8 ng/ μ l onto anti hIgG Fc capture (AHC) sensors. F4-Fabs were biotinylated and then loaded onto SA sensors (ForteBio) at a concentration of 16 ng/ μ l. After loading of the sensors with the analyte, sensors were dipped into GP_{SUDV} (courtesy of John Dye) at indicated concentrations. Data was globally fitted and K_D values were determined.

3. Results

3.1. Degradation of F4 within cellular endosomes

The X-ray structure of SUDV GP in complex with 16F6 (the murine parent of F4) reveals that the binding site is similar to that of KZ52 on EBOV [12]. The structure of F4 has not been determined, but competition ELISA suggests that it binds in the same region as 16F6, consistent with the intended design [5]. mAb binding to GP presumably occurs at the viral surface, but viral membrane fusion occurs deep within the endocytic pathway [27]. Thus, in order to block membrane fusion-associated GP transitions, it is likely that F4 colocalizes to endosomal compartments along with viral particles. To explore intracellular trafficking of F4-virus complexes, we utilized vesicular stomatitis virus particles bearing the GP of SUDV (Boniface variant), VSV-GP_{SUDV}, labeled with a fluorescent neogreen (neo) core [6]. Previous studies have shown that VSV-GPs function as effective surrogates for the investigation of ebolavirus cell entry in Biosafety level 2 (BSL 2) conditions, despite divergent particle morphologies of VSV and ebolaviruses [5,6,11,18,22].

Biolayer interferometry (BLI) experiments showed that antibody binding to the glycoprotein occurs at a k_{on} rate of 1.1×10^4 (1/ms) (Supplementary Fig. 1). To test if the mAb is co-internalized with the virus into the cell, we directly labeled F4 with an Alexa-Fluor 647 dye (AF647) to track the antibody on the surface of the virus. neoVSV-GP_{SUDV} were pre-incubated with AF647-labeled F4 (F4^{AF647}) for 30 min and then added to Vero cells to record the uptake of F4^{AF647}-neoVSV-GP_{SUDV} complexes by spinning disc live cell microscopy (Fig. 1A-C). We observed the uptake of the first recordable particles within 20 min post-addition to the cells (Fig. 1C). Both the viral particles and mAbs had the same trajectory assuming that most of the mAb has been co-internalized with virus. Interestingly, live cell imaging revealed that the antibody has almost been completely degraded inside the endosome after 62 min post-infection, recorded as a loss of the F4^{AF647} signal (Fig. 1B/C). Here, we observed that the degradation in a time-dependent fashion (Fig. 1B; blue dots). Degradation was further confirmed by a series of experiments where we recorded the fate of unlabeled F4 after different time points in fixed Vero cells. For the detection of the antibody, we used a secondary antibody that detects the constant region of the IgG1 portion of F4. Results from this experiment confirmed the observations from the live cell imaging where F4 could not be detected colocalized with neoVSV-GP_{SUDV} after 60 min of incubation (Fig. 1C, left panel). Together, these results suggest that the high proteolytic activity of the endolysosomal milieu is triggering the degradation of the mAb to a certain degree.

To further assess the dependence of F4 degradation on specific endoproteases, we repeated the colocalization of unlabeled F4 with neoVSV-GP_{SUDV} in the presence of a small molecule protease inhibitor. Degradation of the mAb was blocked by the pre-treatment of the Vero cells with E64d, a cysteine protease inhibitor specific for cathepsins (Fig. 1D; right panel). Even one hour of after addition of the F4-neoVSV-GP_{SUDV} complexes, most of the mAb was detectable and colocalized with virus. Thus, it seems that cathepsins play a major role in the degradation of F4 inside the endosome. To test whether the phenomenon of blockage of degradation by E64d is also universally applicable to other mAbs, we tested several EBOV-specific antibodies, including ZMapp™ cocktail components by a similar analysis with neoVSV-GP_{EBOV}. We found that the degradation of 2G4, 4G7, KZ52 and 13C6 is all similarly dependent on endosomal cathepsins (Supplementary Fig. 2).

3.2. Generation of escape VSV-GP_{SUDV} escape mutants

Our previous experiments used competition ELISAs to investigate the binding site of F4, where it was shown that F4 competes with 16F6 binding [5]. By analogy to the 16F6-GP_{SUDV} X-ray structure, the binding of F4 is predicted to bind at the base of GP near the interface of GP1 and GP2. However, the precise contact residues for F4, and whether they differ from that of 16F6, have not yet been investigated. We therefore generated escape mutants against F4 and 16F6 by using a recombinant VSV-GP_{SUDV} (rVSV-GP_{SUDV}). The rVSV-GP_{SUDV} was grown in the presence of F4 or 16F6 for several generations, with the application of incrementally increasing F4 and 16F6 concentrations. A single viral escape mutant bearing substitution of a glycine at position 557 to a glutamic acid (G557E in the N-heptad repeat region (NHR) of GP2, Fig. 2) was isolated against both mAbs. Detailed analysis of the 16F6-Fab X-ray structure in complex with GP_{SUDV} revealed that G557 is in close proximity to the CDR-H3 region of the mAb. The distance of G557 Ca to the Ca of

G102 in CDR-H3 of 16F6 is 3.9 Å. Thus, the substitution of G557E is likely to impair interactions between F4 and/or 16F6 CDR-H3 *via* steric occlusion. Five other mutations in GP1 were also identified in the viral escape mutant, but none of these were located proximally to the 16F6 binding site: E71Q, V751, V96G, E103K, and G131S. While we cannot rule out that these GP1 mutations contribute to the viral escape, the isolation of the G557E mutation in GP2 is suggestive that 16F6 and F4 bind similarly as designed.

3.3. Contributions of the Fc region to virus neutralization and *in vivo* protection

The likely mechanism of neutralization by F4 is sequestration of GP from the structural transformations required for virus membrane fusion. However, the extent to which bivalency and Fc are required, for example for intraspine cross-linking or for “steric” blockade, have not been previously explored. To test whether the constant region of F4 is needed for neutralization, we generated IgG1 truncations with different configurations in the Fc portion of the antibody (Fig. 3). Furthermore, the requirement for bivalency in binding was explored with the F4-Fab. Interestingly, we found that the neutralization potency depends on the length of the constant region while binding affinity of F4-IgG and F4-Fab is similar by BLI (Supplementary Fig. 1). The neutralization efficacy decreases from F4-IgG1 > F4-CH2 > F4-(Fab)₂ > F4-Fab with measured IC₅₀ values of 0.1 nM, 0.7 nM, 2 nM, and 17 nM, respectively. Neutralization efficiency of the F4-IgG1 in comparison with the F4(Fab)₂ clearly shows that the CH2 and CH3 domains are important domains during neutralization since their depletion significantly decreases neutralization. Nonetheless, the Fab and other fragments retain some degree of neutralization potential and thus an aspect of inhibition of viral entry likely involves sequestering the glycoprotein by mAb binding.

Finally, the extent to which Fc-mediated function is important to *in vivo* activity has not been previously explored for F4. For this, we generated two “Fc null” mutants, F4-LALA and F4-N297Q, and performed *in vivo* mice studies with authentic viruses under BSL4 conditions (Fig. 4). A double Leu- > Ala mutation in Fc (L234A, L235A, or LALA) has previously shown to weaken human Fc receptor binding, ADCC triggering, and complement activation [28]. Furthermore, the binding of IgG1 to FcγR is highly dependent on the glycosylation at position 297. Thus, mutation of the critical N-linked glycosylation site in the Fc (N297Q) to produce an aglycone variant has also been shown to drastically reduce FcγR binding. Consistent with this, we observed that both “Fc null” variants of F4 (F4-LALA and F4-N297Q) exhibited ~10-fold reduced binding capacity to TZM-bl cells that stably express the FcγRI relative to WT F4 (Supplementary Fig. 4). However, both Fc null mutants exhibited strong binding and neutralization potential for GP_{SUDV} (Supplementary Fig. 3).

To explore the *in vivo* requirements of Fc function, human lethal WT SUDV (Boniface) was used to challenge 4–6 week old C56BL/6 mice that were deficient in type 1 α/β interferon receptors (IFNAR^{-/-}), an accepted model for lethal SUDV infection [5,11]. The mice were then treated with WT or Fc null mutant F4 at days 1 and 3 post-infection. Notably, this virus and antibody dosing has previously been shown to differentiate protective and non-protective mAbs [6,11,29]. Eight days post-infection, 60% of the animals in the control group died (Fig. 4A). Only one mouse died in the cohort that was treated with F4-N297Q while all

other groups showed 100% survival. The mice treated with F4-N297Q gained 50% weight during infection (Fig. 4B), whereas mice treated with F4 and F4-LALA gained less weight. Negative control (Z.6D8) treated mice experienced weightloss of > 20% as an aggregate throughout days 8–10 post-infection, consistent with hallmarks of SUDV-induced disease. Those mice in the Z.6D8 control group that survived gained weight outside of this 10-day range. Since all mice in this experiment were weighed as a collective average (all mice in the same pan) for convenience within the BSL4 environment, the weightloss data are not amenable to biostatistic analysis. Nonetheless, the aggregate average data illustrate that all treated groups (F4, F4-N297Q, and F4-LALA) experience no weightloss during the challenge experiment, further indicating protection from viral disease by all three F4 variants.

The surviving mice from each cohort were rechallenged with SUDV (but in the absence of mAb therapy) 30 days after the initial challenge to examine if memory immunity had developed. As shown in Fig. 4C, mice treated with F4 WT as well as Fc null mutant developed memory immunity and thus were not susceptible lethal SUDV challenge. The surviving mice were older in this rechallange experiment; as shown with an untreated control group, SUDV is not uniformly lethal to mice at this age. Nonetheless, control group exhibited substantial weightloss within the first 10 days of the rechallange experiment, an indication of illness (Fig. 4D). However, the surviving mice from the F4 and F4 Fc-null treatment groups exhibited no such weight loss.

4. Discussion

We here provide evidence that F4 is internalized along with virus, suggesting that the binding of the antibody likely inhibits the fusogenic triggering of the glycoprotein in endosomes [22]. In previous live cell imaging experiments, it was shown that anti-EBOV antibodies block or delay the actual fusion event inside the endosome [22]. Dequencing experiments with the ZMapp™ components demonstrated that lipid mixing is drastically reduced while viral internalization and trafficking is unaltered [22]. We observed that the majority of the F4 mAb was degraded within an hour of uptake, and that mAb degradation could be mitigated by the addition of protease inhibitors. These results further support the notion that F4 likely inhibits viral entry within cellular endosomes, where viral membrane fusion occurs. However, the timing suggests a short window for neutralization exists, since significant amounts of the F4 mAb were depleted within one hour of uptake. Notably, the median timeframe for fusion of viral and host membranes within endosomes was estimated to be ~45 min for single VSV-GP_{EBOV} particles [22].

We further explored the role of the Fc regions on the neutralization and *in vivo* protection using variants that either lacked the Fc or contained null mutations. *In vitro* results suggest that steric blockage of GPs may be important as bivalent F4 fragments containing incrementally smaller moieties C-terminal to the hinge region had lower activity. Nonetheless, F4 Fab and other fragment retained some level of neutralizing potential indicating that binding and sequestration of GP is sufficient to inhibit viral entry to some extent. Our observation agrees with a previous study that shows that the full-length IgG1 for KZ52 is a more potent neutralizer than the Fab-KZ52 [24]. Fc null mutants F4-N297Q and

F4-LALA provided high levels of protection and memory immunity in a murine challenge model, indicating that, at least for this SUDV-specific mAb, Fc-related functions may not play a critical role in protection. This supports an *in vivo* mechanism whereby protection by F4 is afforded by preventing viral infection and spread, rather than by Fc-mediated clearance of virus or infected cells. This contrasts with previous studies showing that broadly neutralizing HIV-1 antibodies rely on downstream effector functions to minimize the viral load during infection [28]. Furthermore, studies with EBOV-specific mAb suggest that others may require Fc-related function (such as ADCC). Notably, only two of the three ZMapp components (2G4 and 4G7) exhibit neutralizing activity, whereas the third (13C6) does not. Potentially, 13C6's role may require Fc function.

In summary, this study suggests that F4 is taken up into cellular endosomes along with viral particles where it exhibits its neutralization effect. Potency of inhibition of GP_{SUDV}-mediated cell entry is dependent upon the presence and number of the Fc constant domain. However, *in vivo* protection in a mouse model was not dependent on fully functional Fc region. These results provide novel information to understand the mechanisms of SUDV immunotherapy in detail.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Opinions, conclusions, interpretations, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army. The mention of trade names or commercial products does not constitute endorsement or recommendation for use by the Department of the Army or the Department of Defense.

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References

- [1]. Pavot V, Ebola virus vaccines: where do we stand? Clin. Immunol 173 (2016) 44–49. [PubMed: 27910805]
- [2]. de La Vega MA, Stein D, Kobinger GP, Ebolavirus evolution: past and present, PLoS Pathog 11 (2015) e1005221. [PubMed: 26562671]
- [3]. Okware SI, Omaswa FG, Zaramba S, Opio A, Lutwama JJ, Kamugisha J, Rwaguma EB, Kagwa P, Lamunu M, An outbreak of Ebola in Uganda, Trop. Med. Int. Health 7 (2002) 1068–1075. [PubMed: 12460399]
- [4]. Group PIW, Multi-National PIIST, A randomized, controlled trial of ZMapp for Ebola virus infection, N. Engl. J. Med 375 (2016) 1448–1456. [PubMed: 27732819]
- [5]. Chen G, Koellhoffer JF, Zak SE, Frei JC, Liu N, Long H, Ye W, Nagar K, Pan G, Chandran K, Dye JM, Sidhu SS, Lai JR, Synthetic antibodies with a human framework that protect mice from lethal Sudan ebolavirus challenge, ACS Chem. Biol 9 (2014) 2263–2273. [PubMed: 25140871]
- [6]. Wec AZ, Nyakatura EK, Herbert AS, Howell KA, Holtsberg FW, Bakken RR, Mittler E, Christin JR, Shulenin S, Jangra RK, Bharrhan S, Kuehne AI, Bornholdt ZA, Flyak AI, Saphire EO, Crowe

- JE Jr., M.J. Aman, J.M. Dye, J.R. Lai, K. Chandran, A “Trojan horse” bispecific antibody strategy for broad protection against ebolaviruses, *Science* (2016).
- [7]. Howell KA, Qiu X, Brannan JM, Bryan C, Davidson E, Holtsberg FW, Wec AZ, Shulenin S, Biggins JE, Douglas R, Enterlein SG, Turner HL, Pallesen J, Murin CD, He S, Kroeker A, Vu H, Herbert AS, Fusco ML, Nyakatura EK, Lai JR, Keck ZY, Fong SK, Saphire EO, Zeitlin L, Ward AB, Chandran K, Doranz BJ, Kobinger GP, Dye JM, Aman MJ, Antibody treatment of Ebola and Sudan virus infection via a uniquely exposed epitope within the glycoprotein receptor-binding site, *Cell Rep.* 15 (2016) 1514–1526. [PubMed: 27160900]
- [8]. Holtsberg FW, Shulenin S, Vu H, Howell KA, Patel SJ, Gunn B, Karim M, Lai JR, Frei JC, Nyakatura EK, Zeitlin L, Douglas R, Fusco ML, Froude JW, Saphire EO, Herbert AS, Wirchnianski AS, Lear-Rooney CM, Alter G, Dye JM, Glass PJ, Warfield KL, Aman MJ, Pan-ebolavirus and pan-filovirus mouse monoclonal antibodies: protection against Ebola and Sudan viruses, *J. Virol* 90 (2015) 266–278. [PubMed: 26468533]
- [9]. Furuyama W, Marzi A, Nanbo A, Haddock E, Maruyama J, Miyamoto H, Igarashi M, Yoshida R, Noyori O, Feldmann H, Takada A, Discovery of an antibody for pan-ebolavirus therapy, *Sci. Rep* 6 (2016) 20514. [PubMed: 26861827]
- [10]. Flyak AI, Shen X, Murin CD, Turner HL, David JA, Fusco ML, Lampley R, Kose N, Illykh PA, Kuzmina N, Branchizio A, King H, Brown L, Bryan C, Davidson E, Doranz BJ, Slaughter JC, Sapparapu G, Klages C, Ksiazek TG, Saphire EO, Ward AB, Bukreyev A, Crowe JE Jr., Cross-reactive and potent neutralizing antibody responses in human survivors of natural ebolavirus infection, *Cell* 164 (2016) 392–405. [PubMed: 26806128]
- [11]. Frei JC, Nyakatura EK, Zak SE, Bakken RR, Chandran K, Dye JM, Lai JR, Bispecific antibody affords complete post-exposure protection of mice from both Ebola (Zaire) and Sudan viruses, *Sci. Rep* 6 (2016) 19193. [PubMed: 26758505]
- [12]. Dias JM, Kuehne AI, Abelson DM, Bale S, Wong AC, Halfmann P, Muhammad MA, Fusco ML, Zak SE, Kang E, Kawaoka Y, Chandran K, Dye JM, Saphire EO, A shared structural solution for neutralizing ebolaviruses, *Nat. Struct. Mol. Biol* 18 (2011) 1424–1427. [PubMed: 22101933]
- [13]. Bornholdt ZA, Turner HL, Murin CD, Li W, Sok D, Souders CA, Piper AE, Goff A, Shamblin JD, Wollen SE, Sprague TR, Fusco ML, Pommert KB, Cavacini LA, Smith HL, Klempner M, Reimann KA, Krauland E, Gerngross TU, Wittrup KD, Saphire EO, Burton DR, Glass PJ, Ward AB, Walker LM, Isolation of potent neutralizing antibodies from a survivor of the 2014 Ebola virus outbreak, *Science* 351 (2016) 1078–1083. [PubMed: 26912366]
- [14]. Murin CD, Fusco ML, Bornholdt ZA, Qiu X, Olinger GG, Zeitlin L, Kobinger GP, Ward AB, Saphire EO, Structures of protective antibodies reveal sites of vulnerability on Ebola virus, *Proc. Natl. Acad. Sci. U. S. A* 111 (2014) 17182–17187. [PubMed: 25404321]
- [15]. Lee JE, Fusco ML, Hessel AJ, Oswald WB, Burton DR, Saphire EO, Structure of the Ebola virus glycoprotein bound to an antibody from a human survivor, *Nature* 454 (2008) 177–182. [PubMed: 18615077]
- [16]. Nyakatura EK, Frei JC, Lai JR, Chemical and structural aspects of Ebola virus entry inhibitors, *ACS Infect. Dis* 1 (2015) 42–52. [PubMed: 25984565]
- [17]. Hunt CL, Lennemann NJ, Maury W, Filovirus entry: a novelty in the viral fusion world, *Viruses* 4 (2012) 258–275. [PubMed: 22470835]
- [18]. Chandran K, Sullivan NJ, Felbor U, Whelan SP, Cunningham JM, Endosomal proteolysis of the Ebola virus glycoprotein is necessary for infection, *Science* 308 (2005) 1643–1645. [PubMed: 15831716]
- [19]. Malashkevich VN, Schneider BJ, McNally ML, Milhollen MA, Pang JX, Kim PS, Core structure of the envelope glycoprotein GP2 from Ebola virus at 1.9-Å resolution, *Proc. Natl. Acad. Sci* 96 (1999) 2662–2667. [PubMed: 10077567]
- [20]. Harrison JS, Koellhoffer JF, Chandran K, Lai JR, Marburg virus glycoprotein GP2: pH-dependent stability of the ectodomain α -helical bundle, *Biochemistry* 51 (2012) 2515–2525. [PubMed: 22369502]
- [21]. Koellhoffer JF, Malashkevich VN, Harrison JS, Toro R, Bhosle RC, Chandran K, Almo SC, Lai JR, Crystal structure of the Marburg virus GP2 core domain in its postfusion conformation, *Biochemistry* 51 (2012) 7665–7675. [PubMed: 22935026]

- [22]. Spence JS, Krause TB, Mittler E, Jangra RK, Chandran K, Direct visualization of Ebola virus fusion triggering in the endocytic pathway, *MBio* 7 (2016) e01857–01815. [PubMed: 26861015]
- [23]. Shedlock DJ, Bailey MA, Popernack PM, Cunningham JM, Burton DR, Sullivan NJ, Antibody-mediated neutralization of Ebola virus can occur by two distinct mechanisms, *Virology* 401 (2010) 228–235. [PubMed: 20304456]
- [24]. Maruyama T, Rodriguez LL, Jahrling PB, Sanchez A, Khan AS, Nichol ST, Peters CJ, Parren PW, Burton DR, Ebola virus can be effectively neutralized by antibody produced in natural human infection, *J. Virol* 73 (1999) 6024–6030. [PubMed: 10364354]
- [25]. Chandran K, Sullivan NJ, Felbor U, Whelan SP, Cunningham JM, Endosomal proteolysis of the Ebola virus glycoprotein is necessary for infection, *Science (New York, N.Y.)* 308 (2005) 1643–1645. [PubMed: 15831716]
- [26]. Audet J, Wong G, Wang H, Lu G, Gao GF, Kobinger G, Qiu X, Molecular characterization of the monoclonal antibodies composing ZMAb: a protective cocktail against Ebola virus, *Sci. Rep* 4 (2014) 6881. [PubMed: 25375093]
- [27]. Gong X, Qian H, Zhou X, Wu J, Wan T, Cao P, Huang W, Zhao X, Wang X, Wang P, Shi Y, Gao GF, Zhou Q, Yan N, Structural insights into the Niemann-Pick C1 (NPC1)-mediated cholesterol transfer and Ebola infection, *Cell* 165 (2016) 1467–1478. [PubMed: 27238017]
- [28]. Hessel AJ, Hangartner L, Hunter M, Havenith CE, Beurskens FJ, Bakker JM, Lanigan CM, Landucci G, Forthal DN, Parren PW, Marx PA, Burton DR, Fc receptor but not complement binding is important in antibody protection against HIV, *Nature* 449 (2007) 101–104. [PubMed: 17805298]
- [29]. Chen G, Koellhoffer JF, Zak SE, Frei JC, Liu N, Long H, Ye W, Nagar K, Pan G, Chandran K, Dye JM, Sidhu SS, Lai JR, Synthetic antibodies with a human framework that protect mice from lethal Sudan ebolavirus challenge, *ACS Chem. Biol* 9 (2014) 2263–2273. [PubMed: 25140871]
- [30]. Daniela L, Chen G, Maranhao AQ, Rocha LB, Sidhu SS, Piazza RMF, Development and characterization of recombinant antibody fragments that recognize and neutralize in vitro Stx2 toxin from shiga toxin-producing escherichia coli, *PLoS One* 10 (3) (2015) e0120481. [PubMed: 25790467]

Fig. 1.

Live cell imaging of F4-neoVSV-GP_{SUDV} complexes. (A) neoVSV-GP_{SUDV} particles were preincubated with F4^{AF647}, added to Vero cells and then imaged. (B) After 10 min, internalization of the complexes was visualized and recorded over time for 62 min. Events in the magnified box were quantified on the mean fluorescent pixel intensity for the virus (green triangles) and the antibody (blue circles) over time based on the analysis of single images. Representative examples are illustrated in (C). (D) F4-neoVSV-GP_{SUDV} complexes were added to Vero cells. After one hour of incubation, cells were washed, fixed and stained with an anti-IgG-AF555 antibody and imaged (left panel). In the same experiment, Vero

cells were pre-treated for one hour with 300 μ M E64d (right panel). F4-neoVSV-GP_{SUDV} complexes were added for one hour. Cells were washed, fixed and stained with an anti-IgG-AF555 (bar =10 μ m for panels C and D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Fig. 2.
Generation of F4/16F6 escape mutants and analysis of protective proteolytic effects mediated by F4. Crystal structure of the 16F6(Fab)-GP_{SUDV} complex (PDB ID: 3VE0). Insets show proximity of Ca atoms from G557 (GP2 NHR) and G102 (16F6 CDR-H3).

Fig. 3. VSV-GP_{SUDV} *in vitro* neutralization of different F4 variants. Full length F4-IgG1, F4-CH2 lacking the CH3 regions. F4-(Fab)₂ and F4-Fab were compared in a neutralization assay with VSV-GP_{SUDV}.

Fig. 4.

Role of Fc function in *in vivo* protection. Survival (A and C) and weight change curves (B and D) for initial challenge experiment (A and B) and rechallenge of surviving mice (C and D). Mice were provided 500 μ g of mAb therapy at days 1 and 3 post-exposure during the initial challenge. For initial challenge (A and B), mice were 4–6 weeks old whereas mice in the rechallenge experiment were 8–10 weeks old. Untreated group for rechallenge is age matched. Each weight change data point represents the mean of the mice remaining in the group for a given time point. P-values in panel A, relative to Z.6D8 negative control group, were calculated using Fisher's Exact Test with Bonferroni Correction.