THE ANALYSIS OF THE HUMAN ANTIBODY RESPONSE TO FILOVIRUS INFECTION

By

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To survivors of Marburg and Ebola virus infections who generously donated their blood for this study.
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<tr>
<td>MAb</td>
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<td>MARV</td>
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<td>MLD</td>
<td>Mucin-like domain</td>
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CHAPTER I

INTRODUCTION

“In biology, nothing is clear, everything is too complicated, everything is a mess, and just when you think you understand something, you peel off a layer and find deeper complications beneath. Nature is anything but simple.”


Thesis overview

This document is the culmination of my work on characterizing the human antibody response to Marburg and Ebola virus infections. It is divided into five chapters; chapter I provides necessary background information about filoviruses, the structure and function of filovirus glycoprotein, and filovirus entry into the host cell. This chapter also explores the human humoral response against filovirus infections and provides detailed analysis about what we know about the antibodies that target filovirus glycoprotein.

The beginning of my research starts in chapter II. This part of my research focuses on studying the human antibody response to Marburg virus (MARV). I describe a panel of fifty-one human antibodies against MARV proteins that was isolated using peripheral blood B cells from an individual who survived MARV infection several. I use antibodies from the MARV survivor to define the mechanism of MARV neutralization, and provide the evidence that MARV neutralizing antibodies recognize the receptor-binding site of MARV GP.

Chapter III focuses on the nature of cross-reactive antibody response in human survivors of Ebola virus infections. I describe a panel of ninety human monoclonal antibodies to Ebolavirus glycoprotein isolated from survivors of Bundibugyo virus outbreak. I present structural and functional information about human antibodies that neutralize multiple
Ebolavirus species and a cross-reactive antibody that completely protect guinea pigs from the lethal challenge with Ebola virus. In Chapter IV I describe three naturally-occurring human cross-reactive mAbs that target a new antigenic site in the canonical heptad repeat 2 (HR2) region near the membrane proximal region of EBOV GP. I highlight the structural features formed by conserved residues in the protective site that could be used to develop an epitope-based vaccine against infection caused by diverse Ebolavirus species.

In Chapter V I summarize my findings and propose future directions for this work. I believe that understanding the principles underlying molecular recognition of viral protein by potent neutralizing antibodies will have a broad impact on the rational design of therapeutic antibodies and development of vaccines against filoviruses and other emerging viruses.

Epidemiology of Marburg virus infection

Filoviruses are enveloped, negative-sense RNA viruses appearing with a characteristic filamentous shape. The family Filoviridae (order Mononegavirales) can be divided into two major genera: Marburgvirus and Ebolavirus (Kuhn et al., 2013). The genus Marburgvirus includes a single species, Marburg marburgvirus and two divergent viruses: Ravn virus and Marburg virus (MARV).

MARV was discovered in August 1967, when laboratory workers in Marburg and Frankfurt (Germany) and Belgrade (former Yugoslavia, now Serbia) were infected with unknown infectious agent. It was determined that African green monkeys that had been imported from Uganda for the purpose of poliovirus vaccine research were the source of infection. The virus was isolated and characterized by multiple groups and named Marburg virus after the city with the most cases (Kissling et al., 1968; Kunz et al., 1968; Siegert et al., 1968). From 31 infected individuals, seven patients died from complications of the disease (23% mortality rate).
In the following years from 1967 though 1998, only sporadic outbreaks of MARV occurred on the African continent affecting small number of individuals (Brauburger et al., 2012) (Figure 1). In 1987, an isolated case of Marburg disease was identified in Kenya. The virus was isolated and characterized as a new virus in Marburgvirus genus, named Ravn virus (Johnson et al., 1996). The first large outbreak of MARV occurred in 1998-2000 in a gold-mining village of Durba in the northeastern Democratic Republic of Congo (DRC) affecting 154 individuals and killing 83% (Bausch et al., 2006). The outbreak ceased abruptly with the closure of the mine, suggesting that the reservoir of the MARV inhabits mines or caves (Bausch et al., 2006). The largest outbreak of MARV occurred in Angola in 2004-2005 causing 252 cases and 227 deaths (90% mortality) (Towner et al., 2006). In 2007 a small MARV outbreak occurred in western Uganda among miners working in Kitaka Cave. Based on the detection of MARV RNA in bats, virus-specific antibodies in sera, and isolation of MARV virus from bat tissue, the cave-dwelling Egyptian fruit bats (Rousettus aegyptiacus) was identified as a natural host of MARV (Towner et al., 2009). The genome sequences of isolated from miners working in Kitaka Cave and bats from the same cave closely matched (99% identity) (Towner et al., 2009).

While MARV disease remains rare, MARV poses a risk for travelers to Africa. In 2008 two cases of MARV disease were documented in Dutch and American tourists who presumably got infected during a visit to Python Cave in Queen Elizabeth National Park, Uganda. While the Dutch tourist died from MARV disease, the American tourist survived the infection ((CDC), 2009; Timen et al., 2009). In 2012, as I started my thesis studies in the Crowe Lab, I received peripheral blood sample from the American survivor. Using the survivor’s peripheral blood mononuclear cells, I isolated a panel of human neutralizing antibodies against MARV that revealed the mechanism of MARV inhibition (further described in Chapter II). In addition to naturally occurring infections, several cases of
laboratory-acquired MARV infection have been reported in Soviet Union (now Russia) of which one had a fatal outcome (Brauburger et al., 2012; Nikiforov et al., 1994).

Including the recent outbreak in Uganda where 4 individuals died from MARV infection (Albarino et al., 2013), to date there have been in total 467 cases and 372 documented deaths due to the MARV infection. The disease burden of MARV infection in Africa is small when compared to other infections diseases such as HIV (1.3 million deaths caused by HIV in 2009 in sub-Saharan Africa alone, (Brauburger et al., 2012)) and malnutrition. However, there are some studies suggesting that the number of MARV infections is underestimated. During the investigation of a 1998-2000 MARV outbreak in a gold-mining village of Durba in the DRC, medical personal reported that the disease was recognized locally as “syndrome hémorragique de Durba”, occurring as far back as 1980s (Bausch et al., 2006).
Epidemiology of Ebola virus infection

In the *Ebolavirus* genus, there are five virus species, four of which are known to cause severe disease in humans: Ebola virus, Bundibugyo virus, Sudan virus and Taï Forest virus. An additional virus, Reston virus (RESTV) was isolated in 1989 from cynomolgus monkeys imported from Philippines and housed in a quarantine facility in Reston, Virginia, USA. While monkeys developed hemorrhagic disease with high lethality, the small number of confirmed human infections did not result in clinical illness despite the presence of RESTV-specific antibodies in some of the workers handling monkeys (Miranda and Miranda, 2011; Miranda et al., 1991).

Ebola virus first appeared in 1976 when similar cases of hemorrhagic fever emerged in two neighboring locations: first in southern Sudan and then in northern Zaire (now Democratic Republic of the Congo, DRC) (WHO, 1978a, b) (*Figure 2*). The virus was isolated from patients in both outbreaks and named Ebola virus after a small river in northwestern DRC. In the follow up study it was determined that these two outbreaks were caused by two different viruses, Sudan virus (SUDV) and Ebola virus (EBOV) (Cox et al., 1983). The SUDV outbreak resulted in 284 cases with a fatality rate of 53% while EBOV outbreak was responsible for 318 cases with a fatality rate of 89%. It is generally recognized now that within the *Ebolavirus* genus EBOV infection has the highest fatality rates (60-90%), followed by SUDV infections (40-60%) (Feldmann and Geisbert, 2011).

A large outbreak of EBOV occurred in 1995 in the Kikwit town, south of DRC affecting 315 individuals and killing 254 patients (81% fatality rate) (Khan et al., 1999). The Kikwit outbreak has provided much of our current knowledge about EBOV and outbreak management. During the outbreak, 80 (25%) of 315 infected individuals were healthcare personal (Khan et al., 1999).
A large outbreak of SUDV occurred in Uganda in 2000, affecting 425 individuals and killing 173 patients (53% fatality rate) (Lamunu et al., 2004). Between 2001 and 2005 there were five EBOV outbreaks in the Republic of Congo and Gabon resulting in a total of 311 cases and 261 fatalities (84% fatality rate) (Formenty et al., 2006; Leroy et al., 2004; Leroy et al., 2002). The last species of the *Ebolavirus* genus, Bundibugyo virus (BDBV), was discovered in 2007 in Uganda causing 149 cases and 37 deaths (Towner et al., 2008). The Crowe lab obtained de-identified peripheral blood mononuclear cells from 30 survivors of the 2007 BDBV outbreak of Uganda from a repository at Makerere University (Kampala, Uganda) (Towner et al., 2008).
Uganda), which is part of the Walter Reed Army Institute of Research. Antibodies isolated from BDBV survivors are described in Chapter III of this document.

The only case of Taï Forest virus infection occurred in 1995, when a group of scientists was studying a viral hemorrhagic fever epizootic among western chimpanzees (Pan troglodytes versus) in Taï National Park in Ivory Coast (Formenty et al., 1999; Le Guenno et al., 1995). One of the scientists got infected with the virus when she performed necropsies on chimpanzees found dead in the Taï national park. The individual was transported to Switzerland for treatment and later recovered from infection. This was the first case of Ebola hemorrhagic fever reported in West Africa. Until 2013, the Taï Forest virus infection remained the only case of Ebola hemorrhagic fever reported in West Africa.

2013-2015 Ebola outbreak in West Africa

The 2013-2015 Ebola outbreak in West Africa was caused by EBOV Makona variant (Kuhn et al., 2014). The outbreak began in the forested region of southeastern Guinea in December 2013 and then spread into Liberia in March, Sierra Leone in May, and Nigeria in late July 2014. This is the first EBOV outbreak to reach epidemic proportions and the largest EBOV outbreak on record (Briand et al., 2014). The Ebola epidemic has caused more than 28,000 cases and more than 11,000 deaths (WHO Ebola situation report). According to the initial epidemiologic investigation, the suspected first case of the outbreak occurred in Meliandou in Guéckédou prefecture in Guinea where a 2-year old was infected with virus and died on December 6, 2013 (Baize et al., 2014). After a single introduction into the human population, the outbreak was then sustained exclusively by human-to-human transmission (Baize et al., 2014; Gire et al., 2014).

In 2014 a total of 10 individuals with EBOV disease were treated in U.S. hospitals; of these patients, 8 survived (Epstein et al., 2015) (Lyon et al., 2014). The airlifting of infected
individuals for treatment in selected U.S. hospitals has caused an unprecedented level of media attention to EBOV disease, which likely played a role in increasing public concern (SteelFisher et al., 2015).

High-throughput EBOV genome sequencing has played a major role in understanding EBOV evolution and transmission and also informed public health efforts (Baize et al., 2014; Carroll et al., 2015; Gire et al., 2014; Ladner et al., 2015; Park et al., 2015; Simon-Loriere et al., 2015; Tong et al., 2015). Based on the rate of nonsynonymous to synonymous mutations, it was determined that purifying selection becomes increasingly effective over time, removing deleterious mutants from the viral population (Park et al., 2015). Hotspots for non-synonymous divergence have likely resulted from a lower level of constraint on encoded viral proteins (Ladner et al., 2015), as peaks in non-synonymous divergence largely corresponded to intrinsically disordered regions of proteins (for example the region of the glycoprotein gene encoding mucin-like domain) (Olabode et al., 2015). Little evidence was found for EBOV adaptation to the human host suggesting that the frequency of interactions between humans and reservoir hosts may be the dominant factor in controlling the frequency of spillover events (Ladner et al., 2015).

Among survivors of EBOV, chronic health problems, including myalgia, arthralgia, and ocular findings have been considered to be a rheumatologic entity called the post-Ebola syndrome. Recently, the persistence of EBOV in ocular fluid for 9 weeks after the clearance of viremia was reported in EBOV survivor (Varkey et al., 2015). In line with previous reports that EBOV can be cultured from semen samples (Bausch et al., 2007), a recent study has found the first molecular evidence of sexual transmission of EBOV 6 months after patient recovery (Mate et al., 2015), which is more than four times as long as the WHO-defined waiting period for declaring country to be free of EBOV disease.
Filovirus genes and proteins

The family *Filoviridae* belongs to the order of *Mononegavirales*, which contains viruses characterized by a linear, non-segmented, single negative strand of RNA as a genetic material. The EBOV and MARV genomes are about 19 kb in length and they encode seven structural proteins: nucleoprotein (NP), virion protein 35 (VP35), virion protein 40 (VP40), glycoprotein (GP), virion protein 30 (VP30), virion protein 24 (VP24) and RNA-dependent RNA polymerase (L) (*Figure 3*). EBOV but not MARV also encodes one non-structural protein, secreted glycoprotein (sGP). RNA molecule-NP complex is linked to the RNA-dependent RNA polymerase and inner matrix proteins VP30 and VP35 to form a ribonucleoprotein complex, which is engaged in replication and transcription (Volchkov et al., 2001). Both VP24 and VP40 link the nucleoprotein complex with the lipid bilayer of the viral envelope and they are also involved in the formation of nucleocapsid, assembly and budding of viral particles (Leroy et al., 2011). Two matrix proteins, VP35 and VP24 play role in the pathogenicity of filovirus infection as they inhibit host antiviral responses (Leroy et al., 2011).

*Figure 3. Structure of the filovirus virion particle.* The exact position of VP24 in filovirus virions is unclear. From (Kuhn, 2008)
The structure of filovirus glycoprotein

The filovirus glycoprotein (GP) is the only protein on the viral surface. The filovirus GP is a trimer composed of three heavily glycosylated GP1-GP2 heterodimers, which result from furin-like enzyme cleavage of the precursor protein. GP1 and GP2 subunits are linked by a disulphide bond (Volchkov et al., 1998) and mediate all functions necessary for virus to enter the host cell. The GP1 subunit mediates the virus attachment to the cell membrane and receptor recognition. The GP2 subunit anchors the GP spike to the viral membrane and mediates the fusion of viral and host membranes during the viral entry into the host cell.

Crystal structures are available for prefusion GPs of EBOV (Lee et al., 2008a), SUDV (Bale et al., 2012; Dias et al., 2011a), and MARV (Hashiguchi et al., 2015). The crystal structure of EBOV GP in complex with human neutralizing antibody Fab KZ52, illuminated the overall structure of *Ebolavirus* surface protein (Lee et al., 2008a) (Figure 4). The EBOV GP trimer adopts a chalice-like shape, in which GP1 subunits form a bowl encircled by helices of GP2 subunits (Lee et al., 2008a). When the virus is internalized inside the endosome, GP2 unwinds from the GP1 subunit and undergoes a series of conformational rearrangements finally collapsing into a six-helix bundle conformation that initiates the fusion of viral and host membranes (Saphire, 2013). Several structures of post-fusion conformations of the GP2 subunit indicate that pre- and post-fusion structures of GP2 differ significantly (Koellhoffer et al., 2012; Malashkevich et al., 1999; Weissenhorn et al., 1998).

The GP1 subunit can be further divided into several subdomains termed base, head, glycan cap, and mucin-like domain (Saphire, 2013) (Figure 4). The GP1 base forms a hydrophobic concave surface that clamps GP2 subunits (Lee and Saphire, 2009). The GP1 head contains a receptor-binding site topped by a glycan cap and highly glycosylated mucin-like domain (Lee et al., 2008a). The glycan cap and contains four predicted N-linked glycosylation sites and 15-
20 O-linked glycosylation sites. The highly-glycosylated mucin-like domain was excised from GP for crystallization, but a recent study has indicated the relative position of this subdomain using small-angle X-ray scattering (SAXS) in solution (Hashiguchi et al., 2015). The EBOV mucin-like domains extend outwards from the GP core (Hashiguchi et al., 2015) (Figure 5).

Once inside the endosome, GP is cleaved by host proteases that remove approximately 80% of the mass of the GP1 subunit, including the mucin-like domain and glycan cap and exposing the receptor binding site (Chandran et al., 2005; Dube et al., 2009). Both glycan cap and mucin-like domains are dispensable for pseudovirus attachment or entry in cell culture (Yang et al., 2000). It has been suggested the dense clustering of glycans on the glycan cap and mucin-like domain likely shield much of the surface of EBOV GP from humoral immune surveillance, leaving only a few sites on the EBOV GP protein where neutralizing antibodies could bind without interference by glycans (Cook and Lee, 2013).

Figure 4. The structure of Ebola virus glycoprotein. (A) Domain schematic of EBOV GP. White regions indicate crystallographically disordered domains and hash-marked regions indicate construct-deleted regions. GP1 base is green (I), GP1 head - blue (II), GP1 glycan cap - cyan (III), N terminus of GP2 – red, GP2 internal fusion loop – orange, GP2 HR1 – yellow. Red Y-shaped symbols indicate predicted N-linked glycosylation sites. Y-symbols marked with an asterisk were mutated. (B) Molecular surface of EBOV GP viewed on its side (left) and top (right). Monomer A is colored according to the scheme in A. (C) Molecular surface of EBOV GP chalice and cradle. From (Lee et al., 2008a)
GP2 mediates the fusion of viral and host cell membranes and contains the internal fusion loop and heptad repeat regions (HR1 and HR2) (Lee et al., 2008a). The crystal structures of post-fusion GP2 subunit have indicated that HR1 and HR2 form antiparallel α-helices (Malashkevich et al., 1999; Weissenhorn et al., 1998).

Recently, a crystal structure of Marburg virus GP in complex with a neutralizing antibody MR78 isolated from a human survivor of Marburg infection has been reported (Hashiguchi et al., 2015). SAXS data collected for full-length EBOV and MARV GPs indicated that while mucin-like domain of EBOV projects more upward, mucin-like domain of MARV is less upward, potentially covering the sides of the GP trimer (Figure 5). While the glycan cap and mucin-like domain shield the EBOV receptor-binding sites from humoral immune surveillance (Lee et al., 2008a), new SAXS data suggested that the receptor binding site on MARV GP surface might be accessible for antibody binding (Hashiguchi et al., 2015).

Figure 5. Molecular envelopes of full-length filovirus glycoproteins. Envelopes of mucin-like domain-containing MARV GP (A) and EBOV GP (B) ectodomains determined by Small-Angle X-ray Scattering in solution. Rendered Gaussian distributions of molecular envelopes are shown in gray, crystal structures of cleaved MARV GP (that lacks the glycan cap and mucin-like domain, colored in purple and grey) and EBOV GPΔMuc (that lacks the mucin-like domain, colored in blue and grey) are shown as ribbon models. From (Hashiguchi et al., 2015)
Entry of filoviruses

During viral entry, the mucin-like domain and glycan cap that contain N-linked and O-linked glycans mediate binding to host attachment factors present on the cell membrane, including C-type lectins (DC-SIGN, L-SIGN and mannose-binding lectin) (Alvarez et al., 2002; Lin et al., 2003; Marzi et al., 2004; Marzi et al., 2007). New attachment factors that bind to phosphatidylserine present in the EBOV and MARV viral envelope were identified (Kondratowicz et al., 2011). These phosphatidylserine receptors include members of the T-cell immunoglobulin and mucin domain protein (TIM) family and TAM family of receptor tyrosine kinase (Tyro3/Axl/Mer) (Kondratowicz et al., 2011; Moller-Tank and Maury, 2014; Shimojima et al., 2006). Additional study determined that TIM family receptors can bind to phosphatidylethanolamine, which is also present in the virions of filoviruses (Richard et al., 2015) (Figure 6).

Following attachment, filovirus virions are internalized predominantly through macropinocytosis (Nanbo et al., 2010; Saeed et al., 2010). This process is characterized by the actin-mediated membrane ruffling and blebbing followed by the formation of macropinosomes (Mercer and Helenius, 2009). Internalized virions first distribute into early endosomes and then traffic to the late endosomes/lysosomes in a Rab5/Rab7 GTPase-dependent manner (Saeed et al., 2010). Inside the endosome, GP is cleaved by cysteine proteases cathepsin B (CatB) and cathepsin L (CatL) at or around residue 190 (Dube et al., 2009). The cleavage event removes approximately 80% of the mass of the GP1 subunit, including the mucin-like domain and glycan cap (Chandran et al., 2005; Dube et al., 2009).

After cleavage of GP in the endosome, the receptor-binding sites on 19-kDa GP become exposed (Chandran et al., 2005), and the GP1 head then is able to bind to its receptor, Niemann-Pick C1 (NPC1) protein (Carette et al., 2011; Côté et al., 2011) (Figure 6). NPC1 is a large thirteen-pass membrane protein that is expressed inside late
endosomes/lysosomes in all cells and functions as a cholesterol transporter (Carstea et al., 1997). Mutations in \textit{NPC1} gene in humans cause a Niemann-Pick type C disease, a rare but fatal neurovisceral disorder, characterized by accumulation of cholesterol inside late endosomes-lysosomes (Carstea et al., 1997). Fibroblasts obtained from patients with Niemann-Pick disease are resistant to EBOV infection (Carette et al., 2011). Chinese hamster ovary-derived cells deficient in NPC1 were completely resistant to viral infection (Carette et al., 2011; Cote et al., 2011). In mouse models of MARV and EBOV infections, heterozygosity for \textit{NPC1} protects animals from lethal filovirus infection (Carette et al., 2011). A single mutation in \textit{NPC1} gene was found in straw-colored fruit bats that are resistant to EBOV infection (Ng et al., 2015). The reported amino acid change decreased the affinity of NPC1 protein to EBOV GP revealing host adaptation to reduce filovirus replication (Ng et al., 2015). Filovirus entry does not require the full-length NPC1 protein, as a single luminal domain C of NPC1 mediates the filovirus entry into the host cell, and NPC1 domain C linked to synthetic membrane protein constituted a receptor for filovirus infection (Miller et al., 2012).

But what is the function of NPC1 in filovirus entry into the host cell? Several models have been proposed to explain the role of NPC1 during the filovirus entry into the host cell (White and Schornberg, 2012). Initial studies proposed that NPC1 triggers the fusion activity of primed GP (Cote et al., 2011; Miller et al., 2012). However, NPC1 binding is not sufficient to trigger the conformational rearrangement, and the additional factors required for fusion remains to be identified (Miller and Chandran, 2012; White and Schornberg, 2012). Recently, endosomal calcium channels called two-pore channels were identified as an additional entry factor for filoviruses and were proposed to trigger the conformational rearrangement in NPC1-negative endosomes (Falzarano and Feldmann, 2015; Sakurai et al., 2015). However, a recent study challenged this idea by showing that glycoprotein fusion events occur in NPC1+ endolysosomes (Mingo et al., 2015).
Filovirus membrane fusion events are initiated by conformational changes in GP and are thought to be similar to the fusion processes described for other viral GPs (White et al., 2008). Triggering expose and reposition the heptad repeat I sequence in GP2 subunit, projecting the hydrophobic GP2 fusion loop into the endosomal membrane (Gregory et al., 2014).
This extended GP2 conformation then unwinds and refolds into six-helix bundle, in which HR1 and HR2 are packed together, and fusion loop and GP transmembrane domain meet (Malashkevich et al., 1999; Weissenhorn et al., 1998). The GP2 conformation rearrangement drives viral and cellular membrane mixing and fusion pore formation, releasing the ribonucleoprotein complex into the cytoplasm (Harrison, 2008) (Figure 6).

Human humoral response to filovirus infection

The adaptive immune system contributes to protection against filovirus infections in nonhuman primates and small animal models (reviewed in (Wong et al., 2014)). In accordance with evidence observed in animal models, early upregulation of filovirus-specific antibody responses followed by activation of cytotoxic T cells is associated successful recovery from EBOV infection. In contrast, fatal outcome is associated with impaired antibody responses and early activation of T cells unable to control viral replication (Baize et al., 1999). A follow-study identified asymptomatic infections of EBOV in individuals having direct contact with symptomatic patients. Eleven of 24 asymptomatic individuals developed antibody responses to EBOV antigens, including nucleoprotein and VP40 (Leroy et al., 2000). A large serological survey of rural populations in Gabon found EBOV-specific antibodies in 15% of analyzed individuals (Becquart et al., 2010).

Antigen-specific antibodies persist in humans. Survivors from the 1995 Kikwit outbreak contained EBOV-specific antibodies 2 years after disease onset (Ksiazek et al., 1999a). In another study, antibody responses persisted for 10 years in two survivors of 1976 Yambuku outbreak (Ksiazek et al., 1999b). Survivors from 2000 Gulu outbreak contained SUDV GP-specific antibodies as well as serum-neutralizing activity 12 years after infection (Sobarzo et al., 2013b). Analysis of serum samples collected from SUDV survivors demonstrated the highest number of samples with neutralizing activity 6 months after recovery, with
neutralizing antibodies persisting for prolonged time, even 10 years after infection (Sobarzo et al., 2013a).

A study of immune responses of four survivors of EBOV who received treatment at Emory University Hospital found an extensive T- and B-cell activation in all four patients (McElroy et al., 2015). The frequencies of activated T and B cells were comparable to other acute viral infections, with virus-specific plasmablasts present in the blood even 2 months after disease onset (McElroy et al., 2015).

**Ebola virus-specific antibodies**

Several dozens of EBOV-specific monoclonal antibodies (mAbs) have been described in the literature, including nearly 20 murine mAbs (Holtsberg et al., 2015; Qiu et al., 2012c; Shedlock et al., 2010; Takada et al., 2003; Wilson et al., 2000), 7 macaque mAbs (Keck et al., 2015; Shedlock et al., 2010), and a single neutralizing human antibody KZ52, which was isolated from a phage-display library constructed from survivors of 1995 EBOV Kikwit outbreak (Maruyama et al., 1999). Additionally, several mAbs have been described for related SUDV, including a single neutralizing antibody that was generated in mice (Dias et al., 2011b) and several synthetic mAbs isolated from a focused phage library (Chen et al., 2014).

Most of our knowledge about humoral response against filovirus infections has come from studies of murine mAbs that recognize EBOV GP (Figure 7, Table 1). Several groups isolated EBOV-specific antibodies by infecting mice with replicon systems in which the native surface protein was replaced genetically with EBOV GP (Qiu et al., 2011; Takada et al., 2003; Wilson et al., 2000). After immunization, spleen cells were harvested from mice and fused with myeloma cells followed by hybridoma passaging and antibody purification. Purified mAbs were initially screened in neutralization assays (Qiu et al., 2012c; Shedlock et
al., 2010; Takada et al., 2003; Wilson et al., 2000), and the functional activity for many of them was measured *in vivo* using mouse (Qiu et al., 2012c; Takada et al., 2007; Wilson et al., 2000; Zeitlin et al., 2011), guinea pig (Qiu et al., 2012c; Qiu et al., 2014; Takada et al., 2007) and non-human primate models of EBOV infection (Marzi et al., 2012; Olinger et al., 2012a; Pettitt et al., 2013; Qiu et al., 2012b; Qiu et al., 2014; Qiu et al., 2013) (Table 1). In parallel, antigenic sites for several EBOV GP-specific antibodies were determined using X-ray crystallography (Bale et al., 2012; Dias et al., 2011a; Lee et al., 2008a; Olal et al., 2012), negative stain single-particle electron microscopy (Murin et al., 2014), alanine-scanning mutagenesis (Davidson et al., 2015), or linear peptide dot blots (Qiu et al., 2011; Wilson et al., 2000) (Table 1). Results from those studies revealed that the highly-glycosylated mucin-like domains shield the EBOV receptor-binding sites from humoral immune surveillance. As a result, mouse antibodies were found to target the major antigenic regions on the surface of EBOV GP: the mucin-like domain, the glycan cap, and the GP base (Davidson et al., 2015; Murin et al., 2014) (Figure 7).

![Figure 7. Sites of vulnerability on EBOV GP for protective mAbs.](image-url) MAbs target the main three sites on GP surface: mucin-like domain, glycan cap and GP base near GP1/GP2 interface. A monomer of GPΔMuc(PDB ID code is 3CSY) is fit into the core GP of the c13C6:c4G7 complex. MPER – membrane proximal external region. From (Murin et al., 2014)
1. Antibodies to the mucin-like domain

Several mouse antibodies were identified that bind to linear polypeptide sequences between glycans in EBOV mucin-like domains (MLD), including mAbs 6D8, 13F6 and 14G7 (Lee et al., 2008c; Olal et al., 2012; Wilson et al., 2000) (Table 1). Those mAbs protect mice from lethal EBOV infection but fail to neutralize EBOV in vitro (Olal et al., 2012; Shedlock et al., 2010), likely because mucin-like domains, as well as mAbs attached to them, are removed by host cathepsins inside the endosome (Chandran et al., 2005). It has been proposed that the protective effect of the MLD-specific antibodies likely results from the ability of antibodies to recruit immunological effector function rather than preventing binding of virus to the target cell membrane (Olal et al., 2012). Despite the good performance of mAbs specific for the MLD in a mouse model of EBOV infection, both 6D8 and 13F6 failed to protect guinea pigs and non-human primates from lethal EBOV infection when tested individually (Qiu et al., 2014) (Table 1).

2. Antibodies to the glycan cap

MAb 13C6 (the key component of MB-003 and ZMapp cocktails) and 1H3 (the key component of ZMab cocktail) were initially isolated in mice (Qiu et al., 2011; Wilson et al., 2000) and later shown by negative-stain single-particle EM and alanine-scanning mutagenesis to target a second antigenic region – the glycan cap (Davidson et al., 2015; Murin et al., 2014). Both 1H3 and 13C6 weakly neutralize EBOV (IC_{50} > 1.0 μg/mL) (Qiu et al., 2012c; Qiu et al., 2014; Wilson et al., 2000) and protected mice against EBOV (Qiu et al., 2012c; Wilson et al., 2000). Despite the high protective efficiency of glycan-cap specific mAbs in mice, 1H3 failed to protect guinea pigs from lethal challenge (all 5 animal died)
Table 1. Protective mAbs against *Ebolavirus* glycoprotein

<table>
<thead>
<tr>
<th>MAb</th>
<th>Isolated from</th>
<th>Neutrlizing?</th>
<th><em>Ebolavirus</em> species(^a)</th>
<th>GP forms(^b)</th>
<th>Antigenic regions</th>
<th>In mice(^c)</th>
<th>In guinea pigs</th>
<th>In NHPs</th>
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<th>Epitope information</th>
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<td>Yes</td>
<td>Z</td>
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<td>Base</td>
<td>4/5</td>
<td>0/4</td>
<td>(Maruyama et al., 1999)</td>
<td>(Davidson et al., 2015; Lee et al., 2008a)</td>
<td>(Oswald et al., 2007; Parren et al., 2002)</td>
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<td>1H3</td>
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<td>Yes</td>
<td>GP, sGP</td>
<td>Glycan cap</td>
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<td>(Qiu et al., 2011)</td>
<td>(Davidson et al., 2015; Murin et al., 2014)</td>
<td>(Qiu et al., 2012c)</td>
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<td>Z, S</td>
<td>GP, sGP</td>
<td>Glycan cap</td>
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<td>1/3</td>
<td>(Wilson et al., 2000)</td>
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<td>3/5</td>
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<td>3/5</td>
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<td>GP</td>
<td>Mucin-like domain</td>
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<td>1/6</td>
<td>0/3</td>
<td>(Wilson et al., 2000)</td>
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<td>0/3</td>
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<td>(Davidson et al., 2015)</td>
<td>(Qiu et al., 2014; Wilson et al., 2000)</td>
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<td>GP, sGP</td>
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<td>1/3</td>
<td>(Takada et al., 2003)</td>
<td>(Takada et al., 2007)</td>
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<tr>
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<td>S</td>
<td>GP</td>
<td>Base</td>
<td>0%(^d) / 90%(^e)</td>
<td>(Dias et al., 2011a)</td>
<td>(Dias et al., 2011a)</td>
<td>(Chen et al., 2014; Dias et al., 2011a)</td>
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\(^a\) Specificity to various *Ebolavirus* species: Z - Ebola virus, S - Sudan virus,
\(^b\) Specificity to various forms of GP: GP - full length GP, sGP - secreted GP, MLD - mucin-like domain
\(^c\) Number of BALB/c mice surviving challenge of EBOV when 100 µg of mAb is administrated on day 1
\(^d\) Percent of SCID mice surviving challenge of SUDV when 100 µg of mAb is administrated on days 5, 10, 15 and 20
\(^e\) Percent of Type 1 IFN α/β R\(^-\) mice surviving challenge of SUDV when 500 µg of mAb is administrated on days -1, 1 and 4
and 13C6 provided very marginal protection in guinea pigs (1 of 6 animals survived) or non-human primates (1 of 3 animals survived) (Qiu et al., 2014) (Table 1). Similar to the mucin-like domain-specific mAbs, it was suggested that the lower in vitro neutralization activity of glycan cap-specific antibodies might be due to the removal of the glycan cap by host proteases (Chandran et al., 2005; Cote et al., 2011; Misasi et al., 2012) inside the endosome before GP engagement with the Niemann-Pick C1 receptor (Carette et al., 2011; Cote et al., 2011).

Murine antibody 226 neutralizes EBOV and protects mice from lethal infection one day before or two days after virus challenge (Takada et al., 2007; Takada et al., 2003). Three neutralization escape mutants with polymorphisms at residues 134, 194 and 199 of GP1 were obtained for mAb 226, suggesting that 226 binds the disordered loop on the surface of GP1, which has been confirmed to be the site of cathepsin cleavage (Lee and Saphire, 2009). MAb 226/8.2 might block cathepsin cleavage, preventing the engagement of GP core with NPC1 receptor.

3. Antibodies to the base region

KZ52 is the only human monoclonal antibody known to bind EBOV GP and represents the group of antibodies that target GP1/GP2 interface at the GP base (Table 1). This antibody was isolated from a phage display library that was constructed from bone marrow RNA obtained from a survivor (Maruyama et al., 1999). KZ52 neutralized EBOV in vitro (Maruyama et al., 1999), protected guinea pigs from lethal challenge (Parren et al., 2002), but failed to protect non-human primates from EBOV infection (Oswald et al., 2007). KZ52 mAb is specific for the three discontinuous regions of GP1 and GP2, including the internal fusion loop (Lee et al., 2008c). KZ52 neutralizes EBOV most likely by inhibiting the conformational changes required for fusion of viral and endosomal membranes (Lee et al., 2008a).
Several murine Abs have also been reported to bind to the base region of EBOV GPs (Dias et al., 2011a; Murin et al., 2014). The ZMapp cocktail is composed of three EBOV glycoprotein (GP)-specific mAbs (designated c13C6, c2G4 and c4G7) that were isolated initially from mice (Qiu et al., 2011; Wilson et al., 2000), chimerized with human antibody constant regions, and then produced in *Nicotiana benthamiana* plants (Qiu et al., 2014). Single-particle EM reconstructions of these mAbs in complex with EBOV surface protein have revealed key sites of vulnerability on the EBOV GP (Murin et al., 2014) (Figure 7). One such site lies within the GP base at the GP1/GP2 interface; two of three mAbs from the ZMapp cocktail (c2G4 and c4G7) bind to overlapping epitopes located in this region.

**Marburg virus-specific antibodies**

Most of our knowledge about humoral response against filovirus infections has come from studies of mAbs that recognize EBOV GP. As the result, there is little information available about human or mouse antibodies to other filoviruses, such as MARV. Two murine Abs that bind the mucin-like domain of MARV GP reduce MARV budding from infected cells in culture, but fail to neutralize virus directly (Kajihara et al., 2012). Polyclonal MARV-specific mAbs protect non-human primates when administrated passively after challenge (Dye et al., 2012). The epitopes recognized by such polyclonal neutralizing mAbs, and the mechanism of neutralization by which these Abs act, are unknown.

Recently, a diverse panel of ten mouse mAbs against MARV has been described (Fusco et al., 2015). Four of ten antibodies bind MARV-specific “wing” epitope on GP2 subunit. The wing region is present only on the surface of MARV GP and is a portion of the mucin-like domain attached to GP2 subunit. GP2-specific mAbs provide 60%-100% protection in mice challenged with MARV (Fusco et al., 2015).
Successful treatment with cocktails of antibodies

While there is no FDA-approved treatment for filovirus infections, several experimental therapeutics against EBOV are being investigated, including small interfering RNAs (Geisbert et al., 2010; Thi et al., 2015), antisense oligonucleotides (Warren et al., 2010; Warren et al., 2015), a nucleoside analog (Warren et al., 2014), therapeutic vaccines (Feldmann et al., 2007; Geisbert et al., 2008), and monoclonal antibody (mAb) cocktails (Olinger et al., 2012a; Qiu et al., 2012b; Qiu et al., 2014). Of these, preliminary treatment studies suggest that the effect of the antibody cocktails exceeded the efficacy and treatment window of other experimental therapeutics described so far (Marzi et al., 2012; Pettitt et al., 2013; Qiu et al., 2012a; Qiu et al., 2014; Zeitlin et al., 2011) (Table 2).

The ZMapp cocktail is composed of three EBOV glycoprotein (GP)-specific mAbs (designated c13C6, c2G4 and c4G7) that were isolated initially from mice (Qiu et al., 2011; Wilson et al., 2000), chimerized with human antibody constant regions, and then produced in Nicotiana benthamiana (Qiu et al., 2014).

<table>
<thead>
<tr>
<th>Table 2. Efficacy of combined mAb treatment in non-human primates</th>
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<tr>
<td><strong>Cocktail</strong></td>
</tr>
<tr>
<td>ZMapp</td>
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<tr>
<td>ZMab</td>
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<tr>
<td>MB-003</td>
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<td>Two mAb cocktail</td>
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CHAPTER II

MECHANISM OF HUMAN ANTIBODY-MEDIATED NEUTRALIZATION OF MARBURG VIRUS

"...the operators offered a side trip, an option, to a place called the Maramagambo Forest, where the chief attraction was a peculiar site that everyone knew as Python Cave. African rock pythons lived there, languid and content, grown large and fat on a diet of bats."

David Quammen, Spillover: Animal Infections and the Next Human Pandemic

Introduction

Most of our knowledge about humoral response against filovirus infections has come from studies of murine Abs that recognize EBOV GP. From those studies, we learned that mouse nAbs preferentially target peptides exposed in upper, heavily glycosylated domains or lower areas (the GP1 base) where rearrangements occur that drive fusion of viral and host membranes (Saphire, 2013). Abs have not been identified that target protein features of the GP1 head subdomain, where the receptor-binding site to NPC1 protein is located. Ab KZ52, the only reported human EBOV GP-specific mAb, was obtained from a phage display library that was constructed from bone marrow RNA obtained from a survivor (Maruyama et al., 1999). KZ52 binds a site at the base of the GP and neutralizes EBOV, most likely by inhibiting the conformational changes required for fusion of viral and endosomal membranes (Lee et al., 2008).

In contrast, very little is known about the mechanisms by which Abs neutralize MARV. Two murine Abs that bound the mucin-like domain of MARV GP reduced MARV budding from infected cells in culture, but failed to neutralize virus directly (Kajihara et al., 2012).
Polyclonal MARV-specific Abs were shown to protect non-human primates when administrated passively after challenge (Dye et al., 2012). The epitopes recognized by such polyclonal nAbs, and the mechanism of neutralization by which these Abs act, are unknown.

In this chapter, I describe the isolation of a large panel of human nAbs from B cells of an individual who contracted MARV infection in 2008 following exposure to fruit bats in the Python Cave in Queen Elizabeth National Park, Uganda. I used Abs isolated from the human survivor to define the molecular basis of MARV neutralization by Abs. The results show that MARV nAbs recognize the NPC1 receptor-binding site of MARV GP, and in some cases also recognize conserved structural features in the equivalent receptor-binding site on EBOV GP.

I acknowledge Alexander Bukreyev’s group for performing neutralization, protection experiments as well as providing help with escape mutants generation, Andrew Ward’s group for studying MARV-specific mAbs by negative-stain single-particle EM, and Erica Saphire’s group for providing MARV GPs.

Isolation of human monoclonal antibodies against Marburg virus

The success of this project depended on the ability to generate large panels of filovirus-specific neutralizing antibodies. The hybridoma generation technique was used successfully in the Crowe lab in the past to generate hundreds of virus-specific cell lines for dengue and influenza viruses (Krause et al., 2011; Smith et al., 2013). From those studies, it was determined that the frequency of antigen-specific memory B cells in a PBMC sample, the initial transformation efficiency, as well as sensitivity and specificity of the screening assays could impact the total number and functional characteristics of the isolated antibodies.
As the hybridoma generation technique has never been used in the past to obtain filovirus-specific human monoclonal antibodies, I decided to test the overall performance of the method by using PBMCs samples obtained from an individual who survived MARV infection several years earlier. To determine whether the individual was able to mount a successful humoral response, I performed an enzyme-linked immunosorbent assay (ELISA) where antibodies form the subject’s serum were tested for binding to MARV proteins. Strong signal was detected for survivor serum but not for control serum, when plates were coated with irradiated cell lysates prepared from MARV-infected cell cultures (Figure 8A).

![Figure 8](image-url)

**Figure 8. Isolation of human mAbs against Marburg virus.** (A) Serum of MARV survivor contains MARV-specific antibodies; plate was coated with irradiated lysates prepared from MARV-infected cells. (B) EBV-transformed B cells secrete MARV-specific antibodies. (C) Mabs that bind to MARV infected-cell lysate but not to GP target MARV internal proteins.
The hybridoma generation protocol developed in the Crowe lab is mostly amenable to isolation of monoclonal antibodies from circulating memory B cells. To show that samples obtained from the MARV survivor contained memory B cells capable of secreting filovirus-specific antibodies, I transformed in total 80 million PBMCs from the subject with Epstein-Barr virus and, after 7 days, I screened supernates from the plates for the presence of MARV antigen-specific B cell lines. I used three different ELISA assays for screening: plates were coated either with recombinant MARV GP expressed in an insect cell line or irradiated MARV virions purified on sucrose gradient or irradiated lysates prepared from MARV-infected cell cultures. Multiple wells with a high signal were observed in all three assays, suggesting that memory B cells capable of secreting antibodies to MARV antigen are present in PBMCs from the MARV survivor and that those cells are transformable by the hybridoma method (Figure 8B).

During the next step of the hybridoma generation protocol, cells from wells with supernates reacting in the MARV antigen ELISAs were fused with myeloma cells using an electrofusion technique. This is the most critical step of hybridoma generation, as the success of the fusion can be impacted by multiple factors, such as the initial transformation efficiency, viability of the myeloma and target B cells, and the total number of antigen-specific B cells in the fusion suspension. In total, I fused 247 antigen-specific B cell lines and, after the cloning, I was able to generate 51 monoclonal hybridomas secreting MARV-specific human mAbs (21% success rate).

Thirty-nine of these mAbs were specific to the MARV GP, while 12 bound to infected-cell lysate but not to GP; these latter mAbs were shown in secondary screens to bind to MARV internal proteins (NP, VP35, or VP40) (Figure 8C).
Neutralization activity

To evaluate the inhibitory activity of the mAbs, we collaborated with Dr. Alexander Bukreyev’s group at UTMB. The Bukreyev Lab performed *in vitro* neutralization studies using a chimeric vesicular stomatitis virus with MARV GP from Uganda strain on its surface (VSV/GP-Uganda). Eighteen of the 39 MARV GP-specific mAbs exhibited neutralization activity against VSV/GP-Uganda (Figures 9A, 10). Of those 18 nAbs, 9 displayed strong (IC$_{50}$ < 10 µg/mL), 8 nAbs displayed moderate (IC$_{50}$: 10-99 µg/mL), and one displayed weak (IC$_{50}$: 100-1,000 µg/mL) neutralizing activity against VSV/GP-Uganda. We also tested the neutralization potency of all nAbs that bound to MARV GP in a plaque reduction assay using
Of 18 Abs that neutralized VSV/GP-Uganda, 10 Abs exhibited neutralizing activity against MARV-Uganda (Figure 9A, 10).

These data suggest that VSV/GP, often used to study neutralizing potency of Abs because of its BSL-2 containment level, is more susceptible to Ab-mediated neutralization than viable MARV. This difference is likely explained by the significantly lower copy number of MARV GP molecules that incorporate into VSV particles compared with the large number of GP molecules on the surface of filovirus filaments (Beniac et al., 2012; Thomas et al., 1985).

Comparison of MARV neutralizing and non-neutralizing antibodies at concentration up to 1.6 mg/mL revealed dose-dependent activity of those mAbs that neutralized. The neutralization activity of nAbs was not enhanced by the presence of complement. As expected, we did not detect neutralizing activity for any of the 12 Abs specific to MARV NP, VP35, or VP40 proteins.

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**Figure 10. Heatmap showing the neutralization potency of MARV GP-specific mAbs.** The IC50 value for each virus-mAb combination is shown, with dark red, orange, yellow, or white shading indicating high, intermediate, low, or no potency, respectively. IC50 values greater than 1,000 µg/ml are indicated by >. Neutralization assays were performed in triplicate.
Recognition of varying forms of GP

To characterize the binding of isolated Abs to recombinant MARV GPs, I performed binding assays using either a recombinant MARV GP ectodomain containing the mucin-like domain (MARV GP) or a recombinant GP lacking residues 257-425 of the mucin-like domain (MARV GPΔmuc). Based on OD405 values at the highest Ab concentration tested (E_max) and 50% effective concentration (EC50), I divided the MARV-GP-specific Abs into four major groups, based on binding phenotype (designated Binding Groups 1, 2, 3A and 3B; Figure 9B and Figure 12). Binding Group 1 mAbs had an E_max to GP < 2 [i.e., these mAbs never exhibited a maximal binding level to MARV GP]; Binding Group 2 mAbs had an E_max to GP > 2, with EC50 for GP < EC50 for GPΔmuc [i.e., these mAbs bound to the mucin-like domain or glycan cap]; Binding Group 3 had an E_max to GP > 2, with EC50 for GP ≈ EC50 for GPΔmuc [i.e., these mAbs bound equally well to full-length and mucin-deleted forms of GP], with the Group 3A mAbs having an EC50 for GP < 0.5 µg/mL and the Group 3B mAbs having an EC50 for GP > 0.5 µg/mL.

Abs that lacked neutralization activity against VSV/GP-Uganda or MARV-Uganda fell principally into Binding Groups 1, 2, and 3A. Interestingly, all VSV/GP-Uganda nAbs displayed a unique binding pattern and segregated into Binding Group 3B (Figure 10). It was interesting that while both mAbs from Groups 3A and 3B bound equally well to the full-length MARV GP and to the GPΔmuc, EC50 values for nAbs from Binding Group 3B were higher than those for non-neutralizing Abs from Group 3A.

Competition-binding studies

To determine whether mAbs from distinct binding groups targeted different antigenic regions on the MARV GP surface, I performed a competition-binding assay using a real-time
Antibodies are assigned to distinct antigenic regions (or functional regions) if they both can bind the antigen. Antibodies are assigned to the same antigenic region if one antibody blocks the binding of another antibody. I tested 18 MARV nAbs from Binding Group 3B, four Abs from Binding Group 3A, and one Ab from Binding Group 2 in a tandem blocking assay in which biotinylated GPΔmuc was attached to a streptavidin biosensor. Abs from Group 1 and the two non-neutralizing Abs from Binding Group 3B did not bind to biotinylated GPΔmuc.
GPΔmuc in the competition assay and were excluded from the analysis. While non-neutralizing Abs from Binding Groups 2 and 3A did not prevent binding of the Binding Group 3B nAbs to GPΔmuc, all nAbs blocked binding of each of the other nAbs to the antigen and segregated into a single competition-binding group (Figure 11). These data suggested that all of the nAbs target a single major antigenic region on the MARV GP surface.
Electron microscopy studies of antigen-antibody complexes

To determine the location of the antigenic region targeted by MARV nAbs, in collaboration with Andrew Ward’s group, we performed negative stain single-particle electron microscopy (EM) studies using complexes of GPΔmuc with Fab fragments of seven nAbs from Binding Group 3B. The EM reconstructions clearly showed that Fab fragments for

**Figure 13. Neutralizing mAbs from a human survivor of MARV bind to the receptor-binding site of GP**

(A) Representative reference-free 2D class averages of the MARV GPΔMuc:MR Fab complexes.

(B) EM reconstructions of seven Fab fragments of neutralizing antibodies bound to MARV GPΔmuc (side views). All seven antibodies target a similar epitope on the top of GP.

(C) These antibodies can be subdivided based on their angles of approach: (1) those that bind toward the top and side of GP1 at a shallow angle relative to the central 3-fold axis (MR72 in red, MR78 in orange, MR201 in yellow, or MR82 in green) and (2) those that bind at a steeper angle toward the top of GP1 (MR191 in cyan, MR111 in blue, or MR198 in purple).

(D) The crystal structure of EBOV GPΔmuc (GP1 in white and GP2 in dark gray) is modeled into the MARV GP density (mesh), and the angles of approach of the neutralizing antibodies are indicated with arrows, colored as in (B). The footprint of the antibodies is indicated by a black circle targeting residues in the putative receptor-binding site (RBS) through a variety of approach angles.
all seven nAbs bind at the top of the GP in or near the NPC1 protein receptor-binding site (Figure 13A,B). The binding pattern of these Abs could be divided further into two major groups based on their relative angle of approach to the GP head domain. MAbs MR72, MR78, MR201 and MR82 bound toward the top and side of GP1 at a shallow angle relative to the central three-fold axis, while mAbs MR191, MR111 and MR198 bound at a steeper angle toward the top of GP1 (Figure 13C,D).

Antibody neutralization escape mutant viruses

As an additional strategy to define residues on MARV GP involved in binding to nAbs, we generated VSV/GP-Uganda variant viruses that escaped neutralization, and then we determined the sequence of the GP of those mAb escape viruses. Vero E6 cells were inoculated with VSV/GP-Uganda in the presence of MR72 or MR78 nAbs. Two escape mutant viruses were isolated: virus variant VSV/GP-72.5 contained three missense mutations in the MARV GP gene (N129S in the putative NPC1 receptor-binding site, S220P in the glycan cap and P455L in the mucin-like domain) and virus variant VSV/GP-78.1 possessed missense mutation C226Y in the glycan cap (Figure 14A). Consistent with the EM data, six of seven nAbs tested displayed a higher level of neutralization activity against the wild-type VSV/GP-Uganda than to the VSV/GP-72.5 or VSV/GP-78.1 escape mutant viruses, suggesting these nAbs recognize MARV GP in a similar fashion (Figure 14B). MAb MR198 exhibited equal neutralization potency against wild-type VSV/GP-Uganda or the two escape mutant viruses (Figure 14B). As all nAbs segregated into one competition group (Figure 11), bound the MARV GP at the NPC1 receptor-binding site (Figure 13), and displayed a similar profile of neutralization of escape mutant viruses (Figure 14), I proposed that blocking of MARV GP binding to NPC1 is the principal mechanism of MARV neutralization by these naturally-occurring human Abs.
Cross-reactive binding of MARV antibodies with EBOV GP

It is surprising that human MARV nAbs recognize the putative NPC1 protein receptor-binding site on GP, since previous studies suggested that the NPC1 protein receptor-binding
site on EBOV GP may be obscured from Ab binding by the presence of the highly glycosylated glycan cap and mucin-like domain (Lee et al., 2008). To determine whether the MARV nAbs we isolated also could bind in a cross-reactive manner to the EBOV GP receptor-binding site, I performed ELISA binding assays using three recombinant forms of MARV and EBOV GPs: full-length GP ectodomain containing the glycan cap and mucin-like domain (designated MARV or EBOV GP), ectodomains lacking residues 257-425 (MARV) or 314-462 (EBOV) of the mucin-like domain (designated MARV or EBOV GPΔmuc) and cleaved GP ectodomains enzymatically treated to remove the mucin-like domain and glycan cap (designated MARV or EBOV GPcl). Three of the MARV nAbs, designated MR78, MR111 and MR191, recognized the EBOV GPcl that lacked the glycan cap and mucin-like domain (Figure 15A). Remarkably, the MARV nAb MR72 bound all three forms of both EBOV and MARV GPs with similar EC$_{50}$ and E$_{max}$ values, indicating that its epitope, and the EBOV receptor-binding site which it likely overlaps, might be partially accessible for Ab binding even in the full-length form (Figure 15A). We tested the breadth of neutralization of MARV nAbs for filoviruses using a panel of different MARV and EBOV isolates. While multiple MARV Abs displayed neutralizing activity towards different MARV strains, MARV nAbs did not exhibit detectable neutralization activity against EBOV or VSV/EBOV (Figure 15B). Structural analysis of MARV and EBOV GP revealed that the glycan cap and mucin-like domain likely obscure the receptor-binding domain in EBOV but not MARV (Hashiguchi T. et al., 2015).

**In vivo testing of Marburg virus antibodies**

In collaboration with Alexander Bukreyev’s group, we tested the *in vivo* protective activity of the mAbs in a murine model using mouse-adapted MARV strain Ci67 (Warfield et al., 2007; Warfield et al., 2009). Inoculation of mice with MARV Ci67 causes clinical disease, and in a proportion of animals causes lethal disease, although typically less than 100%
Figure 15. Breadth of binding or neutralization of human MARV-specific mAbs for diverse filoviruses. (A) A heat map showing the binding in ELISA of neutralizing mAbs from Binding Group 3B to the MARV and EBOV GPs. EC50 value for each antigen-mAb combination is shown, with dark red shading indicating lower EC50 values and orange or yellow shading indicating intermediate or higher EC50 values. EC50 values greater than 1,000 µg/mL are indicated by >. (B) A heat map showing the neutralization breadth of mAbs from Binding Group 3B. The IC50 value for each virus-mAb combination is shown, with dark red shading indicating increased potency and orange or yellow shading indicating intermediate or low potency. IC50 values greater than 1,000 µg/mL are indicated by >.
lethality in mice (Warren et al., 2014). We selected four mAbs among those with the lowest \textit{in vitro} neutralization IC$_{50}$ values: MR72, MR82, MR213, and MR232. The IC$_{50}$ values in neutralization assays with MARV Uganda or mouse-adapted MARV strain Ci67 were comparable (within two-fold). Seven week-old BALB/c mice were injected with 100 µg of antibody by the intraperitoneal route and challenged with 1,000 PFU of Ci67. Twenty four hours later, antibody treatment was repeated. By day 6, all five control mice developed progressive loss of weight and symptoms of the disease, including dyspnea, recumbency, and unresponsiveness, and on days 8 and 9, two animals were found dead and one animal was found moribund and euthanized. The remaining two animals demonstrated recovery by day 11. In contrast, all animals treated with four antibodies survived and did not display elevation of disease score, with the exception of two animals treated with MR72, which showed a transient marginal loss of weight and increase in disease score on days 6-9, which did not exceed 1 (Figure 16). The observed level of protection was remarkable given the relatively modest \textit{in vitro} neutralizing potency of the antibodies.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure16.png}
\caption{Survival and clinical overview of mice treated with MARV mAbs. Groups of mice at 5 animals per group were injected with individual mAbs by the intraperitoneal route twice: 1 h prior and 24 h after MARV challenge at 100 µg per treatment. Untreated animals served as controls. (A) Kaplan–Meier survival curves. (B) Body weight (C) Illness score}
\end{figure}
Discussion

There is an obvious urgent need for prophylactic and therapeutic interventions for filovirus infections given the recurrence of MARV outbreaks including in October 2014 in Uganda and a massive outbreak of EBOV in West Africa in 2013-2015. There is very little information about the structural determinants of neutralization on which to base the rational selection of antibodies, and for MARV there have been no reported human nAbs.

This study reveals that naturally occurring human MARV nAbs isolated from the B cells of a recovered donor principally target the MARV NPC1 protein receptor-binding site, suggesting that a major mechanism of MARV neutralization is inhibition of binding to receptor. Remarkably, some of the isolated antibodies also bound to the EBOV GP. This mechanism of MARV neutralization was unexpected, because previous studies with EBOV showed that the putative receptor-binding domain on GP is obscured on the surface of virions by the presence of the glycan cap and mucin-like domain, only becoming exposed following cleavage by cathepsins in the endosome. These studies suggest that the configuration of the MARV GP differs significantly from that of EBOV GP because the receptor-binding domain must be accessible for immune recognition on MARV GP. Indeed, determination of the structure of the MARV GP and structural analysis of the interaction of mAb MR78 with MARV and EBOV GP molecules shows this to be the case (Hashiguchi et al., 2015).

The information obtained from these studies can be used to inform development of new therapeutics and structure-based vaccine designs against filoviruses. Furthermore, as these nAbs are fully human and exhibit inhibitory activity, they might be useful as a component of a prophylactic or therapeutic approach for filovirus infection and disease. The
challenge studies using mice here show clear evidence of in vivo activity and suggest additional preclinical studies in other species such as guinea pigs and macaques are warranted. Their ability to bind a broad range of MARV isolates indicates that they may offer detection of or efficacy against new viral strains yet to emerge. Although some of these mAbs bind to certain forms of EBOV GP, these antibodies are not likely to be effective against natural Ebola infection because the EBOV receptor-binding site is obscured on the viral surface. However, such mAbs might neutralize EBOV if they could be delivered to the endosome where the EBOV receptor-binding site is exposed following GP cleavage.

Material and Methods

Donor

The donor was an otherwise healthy adult woman who contracted Marburg virus (MARV) infection in 2008 following exposure to fruit bats in the Python Cave in Queen Elizabeth National Park, Uganda. The donor’s clinical course was documented previously (CDC, 2009). Peripheral blood from the donor was obtained in 2012, four years after the illness, following informed consent. The study was approved by the Vanderbilt University Institutional Review Board.

Viruses

MARV strain 200702854 Uganda (MARV-Uganda) was isolated originally from a subject designated “Patient A” during the outbreak in Uganda in 2007 (CDC, 2009; Towner et al., 2009) and underwent 4 passages in Vero E6 cells. MARV strain Musoke (MARV-Musoke) was isolated during the outbreak in Kenya in 1980 (Smith et al., 1982) and passaged 5 times in Vero E6 cells. MARV strain 200501379 Angola (MARV-Angola) was isolated during the outbreak in Angola in 2005 (Towner et al., 2006) and passaged 3 times
in Vero E6 cells. MARV Ravn virus (Ravn) was isolated from a patient in 1987 in Kenya (Johnson et al., 1996) and passaged 4 times in Vero E6 cells. All strains of MARV were obtained originally from the Special Pathogens Branch, U.S. Centers for Disease Control (CDC) and deposited at the World Reference Center of Emerging Viruses and Arboviruses (WRCEVA) housed at UTMB. The recombinant Ebola Zaire strain Mayinga (EBOV) expressing eGFP was generated in our laboratory by reverse genetics (Lubaki et al., 2013; Towner et al., 2005) from plasmids provided by the Special Pathogens Branch at CDC and passaged 3 times in Vero E6 cells. For analysis of antibody binding by ELISA, viruses were gamma-irradiated with the dose of 5 x 10^6 rad. The recombinant VSV in which the VSV GP protein was replaced with that of MARV strain Musoke (VSV/GP-Musoke) or EBOV strain Mayinga (Garbutt et al., 2004) were provided by Dr. Thomas Geisbert (UTMB) and Dr. Heinz Feldmann (NIH), respectively; a similar virus with GP from MARV (strain 200702854 Uganda) was constructed as described below. All work with EBOV and MARV was performed within the Galveston National Laboratory BSL-4 laboratories.

We used a mouse-adapted strain of MARV for testing the effect of mAbs in vivo. The mouse-adapted Ci67 strain of Marburg virus (Warfield et al., 2007) was provided by Dr. Sina Bavari (U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland) and amplified by a single passage in Vero-E6 cells.

Generation of a chimeric strain of VSV in which VSV G protein was replaced with the GP protein of MARV strain Uganda (VSV/GP-Uganda).

The plasmid pVSV-XN2 carrying cDNA of the full-length VSV anti-genome sequence and the support plasmids pBS-N, pBS-L and pBS-P encoding the internal VSV proteins under control of the T7 promoter were kindly provided by Dr. John Rose (Yale University). The plasmid pC-T7, encoding the T7 polymerase, was kindly provided by Dr. Yoshihiro Kawaoka (University of Wisconsin). For generation of the VSV/GP-Uganda construct, Vero
E6 cell monolayers were inoculated with MARV strain 200702854 and total cellular RNA was isolated and reverse-transcribed. MARV GP ORF was PCR-amplified from cDNA using forward primer 5´-CATGTACGACGCTCAACATGAGGACTA-3´ and reverse primer 5´-TCTAGCAGCTCGAGCTATCCAATATATTAGTAAAGATACGACAA-3´ (underlined are MluI and XhoI endonuclease sites, respectively; italicized are the start and the end of MARV GP ORF – direct and complementary sequences, respectively). To replace VSV G with MARV GP, the resulting PCR-product was cloned into pVSV-XN2 using the unique MluI and XhoI endonuclease sites located between the VSV G gene-start and gene-end signals and flanking its ORF, resulting in the plasmid pVSV/GP-Uganda. To recover the recombinant virus, 1 x 10^6 BSR-T7 cells, kindly provided by Dr. Ursula Buchholz (U.S. National Institute of Allergy and Infectious Diseases), were transfected with the following plasmids: pVSV/GP-Uganda, 5 µg; pBS-N, 1.5 µg; pBS-P, 2.5 µg; pBS-L, 1 µg; pC-T7, 5 µg. After 48 hours, transfected BSR-T7 cells were collected with a cell scraper and transferred, along with the supernates, to Vero E6 cell monolayers for amplification of the recovered VSV/GP-Uganda.

Generation of human hybridomas secreting monoclonal antibodies (mAbs)

Peripheral blood mononuclear cells (PBMCs) from the donor were isolated with Ficoll-Histopaque by density gradient centrifugation. The cells were cryopreserved immediately and stored in the vapor phase of liquid nitrogen until use. Previously cryopreserved samples were thawed, and 10 million PBMCs were plated into 384-well plates (Nunc #164688) using: 17 mL of cell culture medium (ClonaCell-HY Medium A, Stemcell Technologies #03801), 8 µg/mL of the TLR agonist CpG (phosphorothioate-modified oligodeoxynucleotide ZOEZOEZEOEZOEZEOEZOT, Invitrogen), 3 µg/mL Chk2 inhibitor (Sigma #C3742), 1 µg/mL cyclosporine A (Sigma #C1832) and 4.5 mL of clarified supernate from cultures of B95.8 cells (ATCC VR-1492) containing Epstein-Barr virus (EBV). After 7 days, cells from each 384-well culture plate were expanded into four 96-well culture plates (Falcon #353072).
using cell culture medium containing 8 µg/mL CpG, 3 µg/mL Chk2i and 10 million irradiated heterologous human PBMCs (Nashville Red Cross) and incubated for an additional four days. Plates were screened for MARV antigen-specific antibody-secreting cell lines using enzyme-linked immunosorbent assays (ELISAs). Cells from wells with supernates reacting in a MARV antigen ELISA were fused with HMMA2.5 myeloma cells using an established electrofusion technique (Yu et al., 2008). After fusion, hybridomas were resuspended in medium containing 100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine (HAT Media Supplement, Sigma #HO262) and 7 µg/mL ouabain (Sigma #O3125) and incubated for 18 days before screening hybridomas for antibody production by ELISA.

Human mAb and Fab production and purification

After fusion with HMMA2.5 myeloma cells, hybridomas producing MARV-specific antibodies were cloned biologically by two rounds of limiting dilution and by single-cell fluorescence-activated cell sorting. After cloning, hybridomas were expanded in post-fusion medium (ClonaCell-HY Medium E, STEMCELL Technologies #03805) until 50% confluent in 75-cm² flasks (Corning #430641). For antibody production, cells from one 75-cm² flask were collected with a cell scraper and expanded to four 225-cm² flasks (Corning #431082) in serum-free medium (Hybridoma-SFM, Gibco #12045-076). After 21 days, supernates were clarified by centrifugation and sterile filtered using 0.2-µm pore size filter devices. HiTrap Protein G or HiTrap MabSelectSure columns (GE Healthcare Life Sciences #17040501 and #11003494 respectively) were used to purify antibodies from filtered supernates. Fab fragments were generated by papain digestion (Pierce Fab Preparation Kit, Thermo Scientific #44985) and purified by chromatography using a two-column system where the first column contained protein G resin (GE Healthcare Life Sciences #29048581) and the second column contained either anti-kappa or anti-lambda antibody light chain resins (GE Healthcare Life Sciences #17545811 and #17548211 respectively).
Expression and purification of MARV and EBOV GPs

Angola strain MARV GP ectodomains, containing the mucin-like domain (MARV GP) or lacking residues 257-425 of the mucin-like domain (MARV GPΔmuc), were used to screen supernates of transformed B cells and human hybridomas separately. Recombinant proteins for Ravn strain cleaved GP, EBOV Mayinga strain GP, EBOV Mayinga strain GPΔmuc and EBOV Mayinga strain cleaved GP were designed and expressed similarly. Large-scale production of recombinant GP or GPΔmuc was performed by transfection of *Drosophila* Schneider 2 (S2) cells with modified pMTpuro vectors, followed by stable selection of transfected cells with 6 µg/mL puromycin. Secreted GP ectodomain expression was induced with 0.5 mM CuSO₄ for 4 days. Proteins were engineered with a modified double strep tag at the C terminus (enterokinase cleavage site followed by a strep tag/linker/strep tag) to facilitate purification using Strep-Tactin resin (Qiagen #2-1201). Proteins were purified further by Superdex 200 size exclusion chromatography in 10 mM Tris, 150 mM NaCl, pH 7.5 (1X TBS).

Lysates of MARV-infected cells

Lysates were prepared as previously described (Ksiazek et al., 1999). Briefly, Vero E6 cell monolayers in 850 cm² roller bottles were inoculated with approximately 10⁶ PFU MARV or EBOV and incubated at 37 °C until partial destruction of monolayer occurred (approximately 9-10 days). Cell monolayers were detached using 3 mm glass beads, and cell suspensions were centrifuged at 16,000 x g for 10 min at 4 °C. Supernates were discarded, cell pellets were resuspended in 10X excess of borate buffer saline (10 mM Na₂B₄O₇, 150 mM NaCl, pH 9.0), and centrifuged at 16,000 x g for 10 min at 4 °C. Supernates were discarded, cell pellets were resuspended in cold 1% Triton X-100 (Fisher Scientific) in borate buffer saline, vortexed and gamma-irradiated on dry ice at 5 x 10⁶ rad.
The lysates were sonicated with a 600 W Tekmar Sonic Disruptor TM600 (Tekmar) using a cuphorn sonicator at maximum power setting and 50% duty cycle for 10 min, centrifuged at 16,000 x g and the supernates aliquotted.

**Screening ELISA**

ELISA plates were coated with lysates of MARV infected cells (diluted 1:1,000 in Dulbecco phosphate buffered saline, DPBS) or recombinant MARV GP or MARV GPΔmuc proteins (20 µg in 10 mL DPBS per plate) and incubated at 4 °C overnight. Plates were blocked with 100 µL of blocking solution/well for 1 h. Blocking solution consisted of 10 g powdered milk, 10 mL of goat serum, 100 mL of 10X DPBS, and 0.5 mL of Tween-20 mixed to a 1 L final volume with distilled water. The presence of antibodies bound to the GP was determined using goat anti-human IgG horse-radish peroxidase conjugated secondary antibodies (Southern Biotech #2040-05, 1:4,000 dilution) and 1-Step Ultra TMB-ELISA substrate (Thermo Scientific #34029), with optical density read at 450 nM after stopping the reaction with 1M HCl.

**Half maximal effective concentration (EC\(_{50}\)) binding analysis**

MARV or EBOV GPs, MARV or EBOV GPΔmuc, or Ravn or EBOV cathepsin-cleaved GPs were coated onto 384-well plates (Thermo Scientific Nunc #265203) in DPBS at 2 µg/mL overnight, then antigen was removed and plates were blocked with blocking solution made as above. Antibodies were applied to the plates at a concentration range of 1.5 µg/mL to 270 ng/mL (Binding Groups #1, #2 and 3A) and 0.1 µg/mL to 10 ng/mL (Binding Group #3B) using three-fold serial dilutions. The presence of antibodies bound to the GP was determined using goat anti-human IgG alkaline phosphatase conjugate (Meridian Life Science #W99008A, 1:4,000 dilution) and p-nitrophenol phosphate substrate tablets (Sigma #S0942), with optical density read at 405 nM after 120 minutes. A non-linear regression
analysis was performed on the resulting curves using Prism version 5 (GraphPad) to calculate EC$_{50}$ values.

**MARV and EBOV neutralization experiments**

Dilutions of mAbs in triplicate were mixed with 150 PFU of MARV or EBOV expressing eGFP in MEM containing 10% FBS (HyClone), 50 µg/mL gentamicin (Cellgro #30-005-CR) with or without 5% guinea pig complement (MP Biomedicals #642836) in a total volume of 0.1 mL, and incubated for 1 hour at 37 °C for virus neutralization. Following neutralization, virus-antibody mixtures were placed on monolayers of Vero E6 cells in 24-well plates, incubated for 1 hour at 37 °C for virus adsorption, and overlayed with MEM containing 2% FBS and 0.8% methylcellulose (Sigma-Aldrich #M0512-1KG). After incubation for 5 days, medium was removed, cells were fixed with 10% formalin (Fisher Scientific #245-684), plates were sealed in plastic bags and incubated for 24 hours at room temperature. Sealed plates were taken out of the BSL-4 laboratory according to approved SOPs, and monolayers were washed three times with phosphate buffered saline. Viral plaques were immunostained with the serum of rabbits that had been hyperimmunized with MARV, or with a mAb against EBOV, clone 15H10 (BEI Resources #NR-12184). Alternatively, following virus adsorption, monolayers were covered with MEM containing 10% FBS and 1.6% tragacanth (Sigma-Aldrich #G1128). After incubation for 14 days, medium was removed, cells were fixed with 10% formalin, plates were sealed in plastic bags, incubated for 24 hours at room temperature, and taken out of the BSL-4 laboratory as above. Fixed monolayers were stained with 10% formalin containing 0.25% crystal violet (Fisher Scientific #C581-100), and plaques were counted.
VSV-MARV and VSV-EBOV neutralization tests

Neutralization assays were performed in triplicate, as described above for MARV and EBOV. Following neutralization, virus-antibody mixtures were placed on monolayers of Vero E6 cells in duplicate, incubated for 1 hour at 37 °C for virus adsorption, and overlayed with MEM containing 2% FBS containing 0.9% methylcellulose. After incubation for 3 days, medium was removed, monolayers were fixed and stained with 10% formalin containing 0.25% crystal violet, and plaques were counted.

Generation and sequencing of VSV/GP-Uganda escape mutants

Vero E6 cell monolayers with two-fold dilutions of mAbs (12.5 – 200 µg/mL) added to the medium were inoculated with 200 PFU of recombinant VSV/GP-Uganda and incubated at 37 °C for 2-4 days. To determine which samples contained live virus, supernates were collected, virus was titrated in Vero E6 cell monolayers under methylcellulose overlay, monolayers were incubated at 37 °C for 3-4 days, and plaques were counted. Supernates with the highest concentrations of mAbs, which were found to contain live virus by plaque titration, were incubated in presence of serially diluted mAbs followed by titration of virus as above. The procedure was performed a total of three times. Escape mutant viruses harvested after the third passage were cloned biologically by plaque purification. For biological cloning, Vero E6 cell monolayers in 24-well plates were inoculated with dilutions of the escape mutant viruses in the presence of the corresponding mAbs (200 µg/mL of MR72 or 100 µg/mL of MR78) and covered with 0.7% low melting temperature SeaPlaque agarose (Lonza #50100). Monolayers were incubated at 37 °C for 6 days, plaques were visualized with 0.01% neutral red aqueous solution (Electron Microscopy Sciences), picked, resuspended in medium and transferred to Vero E6 cell monolayers in 24-well plates in presence of the corresponding mAbs (200 µg/mL of MR72 or 100 µg/mL of MR78) for virus propagation. In 2-5 days, based on the extent of CPE observed, virus was harvested, and
cells were dissolved in Trizol reagent (Life Technologies 315596018). Total cellular RNA was extracted and reverse-transcribed and amplified by PCR with the primers described above for generation of a chimeric strain of VSV. Two overlapping fragments covering MARV GP ORF were PCR-amplified from cDNA using forward primer 5´-CATGTACGACGCGTCAACATGAGGACTA-3´ and reverse primer 5´-ACTAAGCCCTGCTGCCAGGT-3´ or forward primer 5´-ACAACAATGTACCAGGCAA-3´ and reverse primer 5´-TCTAGCAGCTGAGCTATCAATATTTTAGTAAAGATACGACAA-3´, and the nucleotide sequences of the GP ORFs were determined using standard procedures.

Analysis of growth kinetics of VSV/GP-Uganda escape mutant viruses

Vero E6 cell monolayers in 24-well plates were inoculated in triplicate with VSV/GP-Uganda escape mutants or non-mutated virus at an MOI of 0.00025 PFU/cell in the presence of varying concentrations of the corresponding mAbs. Aliquots of medium were collected every 12 hours and frozen for titration at a later time. Titration of virus in aliquots was performed as above, without adding antibodies to the culture medium.

Biolayer interferometry competition binding assay

Biotinylated GP or GPΔmuc (EZ-link® Micro NHS-PEG₄-Biotinylation Kit, Thermo Scientific #21955) (1 µg/mL) was immobilized onto streptavidin-coated biosensor tips (ForteBio #18-5019) for 2 minutes. After measuring the baseline signal in kinetics buffer (KB: 1X PBS, 0.01% BSA and 0.002% Tween 20) for two minutes, biosensor tips were immersed into the wells containing primary antibody at a concentration of 100 µg/mL for 10 minutes. Biosensors then were immersed into wells containing competing mAbs at a concentration of 100 µg/mL for 5 minutes. The percent binding of the competing mAb in the presence of the first mAb was determined by comparing the maximal signal of competing
mAb applied after the first mAb complex to the maximal signal of competing mAb alone. MAbs were judged to compete for binding to the same site if maximum binding of the competing mAb was reduced to <30% of its un-competed binding. MAbs were considered non-competing if maximum binding of the competing mAb was >70% of its un-competed binding. A level of 30-70% of its un-competed binding was considered intermediate competition.

Sequence analysis of antibody variable region genes

Total cellular RNA was extracted from clonal hybridomas that produced MARV antibodies, and RT-PCR reaction was performed using mixtures of primers designed to amplify all heavy chain or light chain antibody variable regions. The generated PCR products were purified and cloned into the pJet 1.2 plasmid vector (Thermo Scientific, #K1231) for sequence analysis. The nucleotide sequences of plasmid DNAs were determined using an ABI3700 automated DNA sequencer. Heavy chain or light chain antibody variable region sequences were analyzed using the IMGT/V-Quest program (Brochet et al., 2008; Giudicelli et al., 2011). The analysis involved the identification of germline genes that were used for antibody production, location of complementary determining regions (CDRs) and framework regions (FRs) as well as the number and location of somatic mutations that occurred during affinity maturation.

Statistical analysis

EC$_{50}$ values for neutralization were determined by finding the concentration of mAb at which a 50% reduction in plaque counts occurred after incubation of virus with neutralizing antibody. A logistic curve was fit to the data using the count as the outcome and the log-concentration as the predictor variable. The results of the model then were transformed back to the concentration scale. Results are presented as the concentration at the dilution
that achieve a 50% reduction from challenge control with accompanying 95% confidence intervals. Each antibody was treated as a distinct analysis in a Bayesian non-linear regression model.

Sample preparation for EM studies

A Ravn strain MARV GP mucin-deleted construct (GP△muc) was produced by stable cell line expression in Drosophila S2 cells, as described above. Human Fab proteins for MARV-specific antibodies were generated as described above. Fabs were added in molar excess to GP△muc and allowed to incubate overnight at 4 °C. Complexes then were purified by Superdex 200 size exclusion chromatography in TBS.

Electron microscopy and sample preparation

A 4 µL aliquot of each complex that had been diluted to a concentration of ~0.03 µg/mL with TBS buffer was placed for 15 seconds onto carbon-coated 400 Cu mesh grids that had been plasma cleaned for 20 s (Gatan), blotted off on the edge of the grid, then immediately stained for 30 s with 4 µL of 2% uranyl formate. The stain was blotted off on the edge of the grid and the grid was allowed to dry. Data were automatically collected with Leginon (Carragher et al., 2000; Potter et al., 1999; Suloway et al., 2005) using a FEI Tecnai F20 electron microscope operating at 120 keV with an electron dose of 30 e⁻/Å² and a magnification of 52,000X that resulted in a pixel size of 2.65 Å at the specimen plane when collected with Tietz CMOS 4k x 4k CCD camera. Particle orientations appeared to be generally isotropic and images were acquired at a constant defocus value of -1.0 µm at 0° stage tilt.
Image processing of protein complexes

Particles were picked automatically using DoG Picker (34) and placed into a particle stack using the Appion software (Lander et al., 2009). Reference-free 2D class averages were generated with the Xmipp clustering 2D alignment software (van Heel et al., 1996) and sorted into an initial 300 classes. Non-GP particles were removed and the stack was further sub-classified into classes with ~100 particles per class in order to generate the final particle stack used for the reconstruction. Various numbers of class averages were chosen to create initial models using EMAN2 common lines software (Tang et al., 2007). A model that best matched its projected classes was then used for refinement against the raw particle stack, imposing C3 symmetry, and the reconstruction was generated with 10 rounds of refinement and increasingly smaller angular sampling rates with EMAN2 (Tang et al., 2007). All model fitting and manipulation was completed using UCSF Chimera (Pettersen et al., 2004).

In vivo testing. The animal protocol for testing of mAbs in mice was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston. Seven-week-old BALB/c mice (Harlan) were placed in the ABSL-4 facility of the Galveston National Laboratory. Groups of mice at 5 animals per group were injected with individual mAbs by the intraperitoneal route twice: one h prior and 24 h after MARV challenge, using 100 µg per treatment. Untreated animals served as controls. For the challenge, mice were injected with 1,000 PFU of the mouse-adapted MARV strain Ci67 by the intraperitoneal route. Animals were weighed and monitored daily over the three-week period after challenge. Once animals were symptomatic, they were examined twice per day. The disease was scored using the following parameters: dyspnea (possible scores 0-5), recumbency (0-9), unresponsiveness (0-5), and bleeding/hemorrhage (0-5); the individual scores for each animal were summarized.
CHAPTER III

CROSS-REACTIVE AND POTENT NEUTRALIZING ANTIBODY RESPONSES IN HUMAN SURVIVORS OF EBOLOVIRUS INFECTION

“The putative index patient was a 26-year-old woman from Kabango village, Kasitu subcounty, in Bundibugyo district. Hunting spears were found at her home, but hunting as a practice was denied.” (Wamala et al., 2010)

Introduction

The ability of mAbs to bind conserved neutralizing epitopes present on the surface of highly variable viral proteins has been documented extensively for HIV (Burton et al., 2012), influenza viruses (Pappas et al., 2014), dengue virus (Rouvinski et al., 2015), paramyxoviruses (Corti et al., 2013), and alphaviruses (Fox et al., 2015). Despite similar requirements for virus entry into the cell (Misasi et al., 2012), GPs from BDBV, EBOV, and SUDV strains differ by over 30% at the amino acid level (Towner et al., 2008). This overall genetic divergence between species of genus Ebolavirus has hampered the development of ebolavirus cross-neutralizing Abs. The key components of multiple antibody cocktails developed over the last decade neutralize only viruses of species Zaire ebolavirus. A weakly neutralizing mAb c13C6 cross-reacts with SUDV GPs (Wilson et al., 2000), but it is unknown whether this mAb neutralizes SUDV. Cross-reactive antibodies in serum can be elicited during natural infection in humans or vaccination of animals. The serum of individuals who survived BDBV, EBOV, or SUDV infections contained ebolavirus cross-reactive IgG but not IgM (Macneil et al., 2011). Other studies demonstrated that mice immunized with a vaccine bearing the GP of EBOV generated cross-reactive polyclonal mAbs to other ebolaviruses such as BDBV and SUDV (Meyer et al., 2015; Ou et al., 2012). Four broadly reactive non-
neutralizing mAbs were isolated in mice after vaccinating animals with recombinant vesicular stomatitis virus (rVSV) expressing EBOV GP and then boosting initial immune response with the heterologous virus containing SUDV GP (Hernandez et al., 2015). The epitopes recognized by such cross-reactive mAbs are unknown.

In this chapter, I describe the isolation of a large panel of BDBV-specific and *Ebolavirus* cross-reactive mAbs from B cells of survivors of BDBV infection. The results show that a large proportion of mAbs with potent neutralizing activity against BDBV bind to the glycan cap and recognize diverse epitopes within this major antigenic site. We identified several glycan cap-specific mAbs that neutralized multiple *Ebolavirus* species and a cross-reactive mAb that completely protected guinea pigs from the lethal challenge with heterologous EBOV when used as monotherapy. Several of these naturally occurring antibodies exhibit the most potent protective capacity reported, and they possessed unprecedented cross-reactivity for multiple *Ebolavirus* species including SUDV for which neutralizing human mAbs have not been reported.

I acknowledge Alexander Bukreyev’s group for performing neutralization, protection experiments as well as escape mutants generation, Andrew Ward’s group for studying EBOV-specific mAbs by negative-stain single-particle EM, Erica Saphire’s group for providing *Ebolavirus* GPs, and Ben Doranz team for performing epitope mapping of selected mAbs using saturation mutagenesis.

### Isolation of Human MAbs

To generate human cell lines secreting human mAbs to BDBV, I transformed peripheral blood B cells from seven survivors of the 2007 Uganda BDBV outbreak with EBV. To determine the breadth of antibody response in survivors of ebolavirus infection, I
screened supernatants from EBV-transformed B cell lines for binding to GPs from diverse representatives of filovirus species: BDBV, EBOV, or MARV (Figure 17). I also used the same GP panel to screen supernatants from transformed B cell lines derived from a survivor of the 2014 EBOV outbreak (Figure 17B) or from a donor who survived MARV infection (Figure 17). I color-coded GP-reactive supernatants based on the cross-reactivity pattern: species-specific cell lines are highlighted in black; cross-reactive lines to 2 or 3 species are shown in yellow or blue, respectively (Figure 17A-C). While approximately half of GP-specific B cell lines obtained from BDBV survivors produced antibodies specific to BDBV GP, 24-50% of GP-reactive B cell culture supernatants also cross-reacted with EBOV GP (Figure 17A, Table 3). Similarly, 36% of GP-specific B cell lines obtained from the EBOV survivor cross-reacted with the heterologous BDBV GP (Figure 17B, Table 3). Despite the apparent presence of B cells encoding cross-reactive antibodies in survivors of BDBV or EBOV infections to GPs from heterologous *Ebolavirus* species, I detected a very limited cross-reactivity with GPs from MARV, which belongs to a different genus in the family *Filoviridae* (Figure 17A, Table 3). In line with this finding, 90% of GP-reactive B cell lines obtained from the MARV survivor reacted with autologous GP, and only 2% of antigen-specific B cell lines produced *Ebolavirus* cross-reactive Abs (Figure 17C, Table 3). The limited cross-reactivity of mAbs to GPs from *Ebolavirus* and *Marburgvirus* species likely is due in part to low sequence conservation between GPs from two genera (only 27% amino acid identity between BDBV and MARV GP) as well as differences in epitope availability caused by different positions of the mucin-like domains on the GP surface of *Ebolavirus* and *Marburgvirus* (Flyak et al., 2015; Fusco et al., 2015; Hashiguchi et al., 2015).
Figure 17. Cross-reactive B cell responses in filovirus immune donors. Supernatants from EBV-transformed PBMC samples isolated from survivors were screened in ELISA binding assays using BDBV, EBOV or MARV GPs (A-C). Results for four BDBV survivors (A), one EBOV survivor (B) or one MARV survivor (C) are shown. Height of the bars indicates OD_{405} nm values in ELISA binding to full-length extracellular domain of GP of the indicated virus species. Reactive supernates are color-coded based on the cross-reactivity pattern: species-specific cell lines are highlighted in black; cross-reactive lines to 2 or 3 species are shown in yellow or blue, respectively.
I fused transformed cells from B cell lines producing BDBV GP-reactive Abs with myeloma cells and generated 90 cloned hybridomas secreting BDBV GP-reactive human mAbs. To determine the breadth of mAb binding, I screened the mAbs in ELISA binding assays using recombinant GPs from multiple filoviruses: BDBV, EBOV, SUDV, or MARV GPs. While 33 Abs recognized only the autologous BDBV GP (designated Groups 1A, 1B), 20 Abs recognized both BDBV and EBOV GPs (Groups 2A, B), and 37 Abs recognized GPs from BDBV, EBOV and SUDV (Groups 3A, B) (Figure 18A, 19A). The relative proportions of antibodies that recognize glycoproteins from 1, 2, or 3 *Ebolavirus* species did not correlate fully with the B cell line frequencies in the initial screen, which can be explained by our prioritization on recovery of a high number of cross-reactive mAbs. I was not able to isolate Abs that bind to the heterologous MARV GP (Figure 18A).

I further characterized the binding of species-specific or cross-reactive mAbs to recombinant GPs by performing a binding assay with the recombinant form of GP that is secreted from the cell to the extracellular space during natural infection (sGP, secreted GP) (Sanchez et al., 1996; Volchkov et al., 1995). While the *Ebolavirus* GP is a trimer, sGP forms dimers in which each protomer shares only the amino-terminal 295 amino acids with GP. The majority of mAbs recognized epitopes shared between BDBV GP and BDBV sGP.

<table>
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<th>Subject</th>
<th>Total number of GP-specific lines</th>
<th>Reactivity of GP-specific B cell lines with diverse GPs (%)</th>
<th>BDBV-specific</th>
<th>BDBV/EBOV cross-reactive</th>
<th>EBOV-specific</th>
<th>BDBV/EBOV/MARV cross-reactive</th>
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Table 3. Percentages of lines secreting antibodies species-specific, or cross-reactive antibodies
Figure 18. Cross-neutralizing antibodies from survivors of natural BDBV infection. (A) Heat map showing the binding of BDBV mAbs to a panel of filovirus GPs. The EC$_{50}$ value for each GP-mAb combination is shown, with dark red, orange, yellow, or white shading indicating high, intermediate, low, or no detectable binding, respectively. EC$_{50}$ values greater than 10,000 ng/mL are indicated by the > symbol. NAb names are highlighted in red. (B) Heat map showing the neutralization potency of BDBV GP-specific mAbs against BDBV. IC$_{50}$ values greater than 10,000 ng/mL are indicated by the > symbol.
Figure 19. Binding and neutralizing activities of BDBV mAbs. (C) Binding of representative mAbs from six distinct binding groups to the filovirus GP. (D) Neutralization activity of representative neutralizing mAbs from three binding groups against BDBV, EBOV or SUDV. Error bars represent the SE of the experiment, performed in triplicate.
BDBV sGP in ELISA (designated Groups 1B, 2B, or 3B) (Figure 18A, 19A). Antibodies from Groups 1B, 2B, or 3B also bound the recombinant GP form that lacks highly glycosylated mucin-like domains (BDBV GPΔmuc), suggesting that mAbs from these three groups target epitopes outside of mucin-like domains (Figure 20).

To evaluate the inhibitory activity of isolated mAbs, in collaboration with Dr. Bukreyev’s group, we tested mAbs in a BDBV neutralization assay. Thirty-one of the 90 BDBV GP-reactive mAbs had half-maximal inhibitory concentration (IC$_{50}$) values < 10 µg/mL, and we defined these as neutralizing antibodies (nAbs) (Figure 18B where nAb names are highlighted in red). Several nAbs displayed an extremely high neutralizing potency, with IC$_{50}$ values below 1 ng/mL (Figure 18B). Eighteen of 31 nAbs bound only to BDBV GP in ELISA, 6 nAbs recognized BDBV and EBOV GPs, and the remaining 7 nAbs bound to GPs from representatives of three Ebola virus species: BDBV, EBOV, and SUDV. These results suggest that cross-reactive mAbs in our panel possess neutralizing activity to multiple ebolaviruses. To test this hypothesis, we screened BDBV425 (a Group 2A nAb) in an EBOV neutralization assay as the nAb with the lowest EC$_{50}$ value to the heterologous EBOV GP, and we determined that BDBV425 neutralized the heterologous EBOV. Encouraged by this result, we tested nAbs from Groups 3A and 3B in EBOV or SUDV neutralization assays to determine whether cross-reactive nAbs can neutralize three Ebola virus species. We found two cross-reactive nAbs from Group 3A (BDBV43 and BDBV324) that neutralized all three Ebola viruses: BDBV, EBOV and SUDV (Figure 19B, BDBV43). The remaining 5 nAbs from Group 3A and 3B neutralized BDBV and EBOV but failed to neutralize SUDV (Figure 19B, BDBV289).
Figure 20. Antibodies from groups 1B, 2B and 3B recognize BDBV GP and BDBV GPΔmuc but not BDBV sGP in ELISA binding assay. The binding of selected antibodies to BDBV GP, BDBV GPΔmuc and BDBV sGP proteins was tested at a single mAb concentration 10 µg/mL.
Major Antigenic Sites Recognized by Human MAbs

To determine whether Abs from distinct binding groups targeted different antigenic regions on the BDBV GP surface, I performed a quantitative competition-binding assay using a real-time biosensor. I tested 4 BDBV nAbs from binding Group 1A, 5 nAbs from binding Group 1B, 4 nAb from Group 3A and 3 nAbs from Group 3B in a tandem blocking assay in which BDBV GP was attached to the biosensor. I also tested 5 non-neutralizing antibodies from Group 1A to determine whether non-neutralizing antibodies target a unique epitope on GP surface.

Non-neutralizing and neutralizing mAbs from Group 1A and nAbs from Group 3A blocked binding of each other to the GP antigen and segregated into a single competition-binding group (Figure 21). These results suggest that mAbs from Group 1A and Group 3A target a single antigenic region that contains epitopes shared between GP and sGP (Figure 18A). NAbs from Group 3B that did not recognize sGP in ELISA (Figure 18A) segregated into a separate competition-binding group. Group 1B antibodies were interesting in that two nAbs in this group competed for binding with Group 3B nAbs, while three nAbs from the group competed for binding with antibodies from Group 3A (Figure 21).

These findings suggest based on competition-binding studies that there are at least two major antigenic regions recognized by human BDBV nAbs. The first major antigenic region contains epitopes which both sGP and GP share (recognized by mAbs from Groups 1A, 3A) as well as epitopes that are present only on the GP surface (recognized by three mAbs from Group 1B). The second major antigenic region contains only epitopes that are present on the GP surface but not sGP (recognized by two mAbs from Group 1B and three mAbs from Group 3B).
Figure 21. BDBV-neutralizing antibodies target at least two distinct antigenic regions of the GP surface. Data from competition-binding assays using non-neutralizing mAbs from binding Group 1A (white background) and neutralizing mAbs from binding Groups 1A, 1B, 3A or 3B (pink background). Numbers indicate the percent binding of second mAb in the presence of the first mAb, compared to binding of un-competed second mAb. MAbs were judged to compete for the same site if maximum binding of second mAb was reduced to <30% of its un-competed binding (black boxes with white numbers). MAbs were considered non-competing if maximum binding of second mAb was >70% of its un-competed binding (white boxes with red numbers). Grey boxes with black numbers indicate an intermediate phenotype (competition resulted in between 30 and 70% of un-competed binding). Blue, purple, and green dashed lines indicate what appear to be major competition groups; the blue and purple groups overlap substantially but not completely.
Diverse Patterns of Molecular Recognition Defined by Negative Stain Electron Microscopy

To determine the location of the two major antigenic regions targeted by human BDBV nAbs, we performed negative-stain single-particle electron microscopy (EM) studies using antibodies from Groups 1A and 1B, in collaboration with Andrew Ward’s group. The EM class averages and reconstructions showed clearly that the two major antigenic regions, defined in competition-binding experiments, corresponded to two distinct sites on GP surface: the glycan cap and the GP base.

Comparison of the structures of glycan cap-directed mAbs from Group 1A with those in Group 1B revealed that the antibodies have partially overlapping epitopes, but approach the glycan cap at distinct angles (Figure 22A, B). We fitted a previously determined atomic resolution structure of Sudan virus (SUDV) GPΔmuc (Bale et al., 2012), which reveals more residues of the glycan cap region than the equivalent EBOV structure, into the envelope of GP from the EM reconstructions and determined the regions targeted by each mAb (Figure 22D, E). BDBV335, which binds GP and sGP equally well, mainly targets a region between residues 274-282. This region appears well defined in the BDBV335 EM map, indicated by the large lobe on the outside of the glycan cap that closely resembles that region in the GP crystal structure. When viewed along the three-fold axis of GP, BDBV41 binds to the right of BDBV335, further up on the glycan cap, close to a loop that extends from residue 266 to 277. BDBV41 also may make contacts with a loop that extends toward the mucin-like domains, from residue 309 to 312 or further in regions that were unresolved in the GP crystal structure. BDBV432 binds to the left of BDBV335, at the top of a helix-loop at residues 259-266, and likely makes extensive contacts with a loop from residues around 302-312. Despite a lack of binding to sGP, BDBV432, as well as BDBV353, bind in the
glycan cap region, suggesting that these mAbs make contacts with residues that are exclusive to GP.

The other antibodies in Group 1B bind an epitope at the base of GP. These antibodies, including BDBV255 and BDBV259, bind further down on GP than has been observed previously with murine mAbs, possibly contacting residues within GP2 that are part of the membrane proximal external region (MPER) (Figure 22C, D, and E). These antibodies were refractory to a reconstruction by EM due to predominant side views of the particles and also apparent flexibility. The class averages, however, clearly show that these antibodies bind an epitope that extends below the base of GP. Three Fabs can be seen in some of the class averages, indicating that despite the apparent small size of this region, three antibodies can be accommodated on one GP trimer. Although the Fabs adopt various positions in each class average, there is not a continuous range of flexibility since the Fabs themselves are well resolved. These antibodies may require the full MPER and transmembrane (TM) regions, as well as a membrane, in order to bind stably. These features are all lacking in the current recombinant protein used here, a soluble form of the extracellular domain of GP. While the GP2 region is well conserved across the filoviruses, these BDBV-specific mAbs likely bind non-conserved regions in GP2 proximal to the TM region.
Figure 22. BDBV-neutralizing antibodies bind to the glycan cap or base region of GP. (A) Shown are negative-stain electron microscopy reference-free 2D class averages of Group 1A antibodies that bind both the glycan cap of GP and sGP, and Group 1B antibodies that bind the glycan cap of GP but not sGP. BDBV GP or GPΔmuc was used to generate complexes. (B) 3D reconstructions of glycan cap binders from Groups 1A and 1B reveal that these antibodies bind the glycan cap at overlapping but distinct epitopes. Top (left) and side (right) views of the complexes are shown. (C) Reference free 2D class averages of Group 1B antibodies (left) reveals that these antibodies bind an epitope below the base of GP that is flexible. In the middle image, GP is colored yellow and each Fab colored green. The right-hand panel illustrates a superimposition of crystal structures of SUDV GPΔmuc (PDB 3VE0) and Fabs (PDB 3C9Y) to demonstrate how Fab may bind to GP. (D) The composite model delineates the epitopes of the glycan cap mAbs in Group 1A or 1B. Side (above) and top (below) views are shown. (E) Docking a crystal structure of SUDV GPΔmuc (PDB 3VE0) (Bale et al., 2012), which contains a more complete model of the glycan cap region targeted by Group 1A/B mAbs, reveals how Group 1A/B mAbs target a broad region in the GP1 centered on the glycan cap, near the beginning of the mucin-like domains. Group 1B mAbs that target the base likely bind to a loop near the membrane proximal external region) that is flexible and has not yet been resolved at high resolution. TM = transmembrane region; CT = cytoplasmic tail.
As the Group 3A (cross-reactive) nAbs competed for binding with Group 1A (BDBV-specific) nAbs (Figure 18), I hypothesized that some structural features of the glycan cap are conserved between GPs from multiple Ebolavirus species. In collaboration with Ben Doranz’s group at Integral Molecular, we sought to identify amino acids that define epitopes for three Group 3 nAbs (BDBV270, BDBV289, and BDBV324) using a comprehensive EBOV GP alanine-scanning mutation library. Epitope mapping identified critical residues for binding by each nAb, W275 for BDBV270, W275 and Y241 for BDBV289, W275 and L273 for BDBV324. Residues for which mutation reduced binding of three nAbs from Group 3A were visualized on the surface of the high-resolution structure of EBOV GP (PDB ID 3CSY). This finding suggests that each of these antibodies recognize overlapping epitopes in the GP glycan cap (Figure 23A, B). The previously described murine nAbs 2G4 and 4G7 and the human nAb KZ52 bind the base region of the GP (Lee et al., 2008a; Murin et al., 2014), and mutations of the W275 or L273 residues did not reduce the binding of these nAbs (Figure 23C). Dr. Bukreyev’s lab tied to passage VSV/BDBV-GP in the presence of BDBV223 or BDBV289 in an attempt to generate escape mutant viruses, but could not isolate neutralization-resistant viruses. An isolate passaged in the presence of BDBV223 with a R574H polymorphism in heptad repeat 1 (HR1) region was identified, and for BDBV289 an isolate with an I584M polymorphism in the HR1 region alone or in combination with an E149K substitution in the receptor-binding domain. However, none of these mutations was associated with the ability of those viruses to resist neutralization by the corresponding mAb.
In collaboration with Andrew Ward’s group, we further characterized the antibody BDBV289 by single particle EM studies of antibody in complex with GP. BDBV289 binds the glycan cap region of GP, centered on the residues W275 and Y241 \((\text{Figure 23D})\). The angle of approach resembles that of the mAb 1H3 from the antibody cocktail ZMab, although 1H3 is specific to EBOV and is weakly neutralizing (Murin et al., 2014; Qiu et al., 2011). Further, BDBV289 also binds sGP, which shares the first 295 amino acids of GP1 with GP, including the glycan cap region (Sanchez et al., 1996; Volchkov et al., 1995). Therefore, despite
previous hypotheses that propose that sGP is an immune decoy and that cleavage of the glycan cap prevents neutralizing antibodies from binding this region (Mohan et al., 2012; Murin et al., 2014), we have now identified several antibodies that challenge these ideas. Interestingly, BDBV289 targets an overlapping epitope with antibodies that we identified to be specific to BDBV and that do not bind sGP (Figure 19). Therefore, the glycan cap region is a major antigenic site that contains epitopes with subtle features that influence sGP and GP binding, neutralization, and species cross-reactivity of targeting mAbs.

Therapeutic Efficacy of Human MAbs in Small Animal Models of EBOV Infection

Finally, in collaboration with Alexander Bukreyev’s group, we set out to test the in vivo efficacy of the cross-reactive nAbs using a guinea pig model of EBOV infection. We selected two nAbs from Groups 3A (BDBV289) and 3B (BDBV223) that bound non-overlapping antigenic regions in the competition-binding experiments (Figure 21). Five to six week-old guinea pigs, strain Hartley, were injected with 5 mg of antibody by the IP route once (day 1) or twice (days 1 and 3) after inoculation with 1,000 PFU of guinea pig-adapted EBOV, strain Mayinga. BDBV223 provided marginal protection, as only 1 of 5 animals survived the lethal challenge (Figure 24). Surprisingly, a glycan cap-specific nAb, BDBV289, fully protected guinea pigs when delivered twice after the virus challenge. The protective efficiency of BDBV289 with a single treatment against a heterologous EBOV (Figure 24, 3 of 5 survived) was higher than the protective efficiency of the equivalent glycan-cap-specific mAb c13C6, a component of the ZMapp cocktail (1 of 6 survived) (Qiu et al., 2014). To determine whether a combination of two mAbs that target two neutralizing epitopes on EBOV GP surface confer better protection than treatment with a single mAb alone, we tested the combination of BDBV223 and BDBV289 in guinea pigs. The combination of two antibodies provided
complete protection against a heterologous EBOV with only a single treatment (Figure 24). We isolated viral RNA from blood of representative animals that were treated with mAbs BDBV223 or BDBV289 but died and then sequenced the genes encoding the GP. Several polymorphisms were detected, but none appeared to be directly related to the epitope specificity of the mAb used for treatment.

Discussion

This study reveals that natural BDBV infection in humans induces the development of ebolavirus cross-reactive antibodies that target epitopes on the GP surface that are conserved in diverse species of genus *Ebolavirus*. During these studies we isolated 90 human mAbs from humans following BDBV infection and found 57 cross-reactive mAbs that recognized heterologous EBOV GPs. Remarkably, some of the isolated cross-reactive mAbs not only bound but also neutralized multiple *Ebolavirus* species. The majority of cross-reactive mAbs neutralized BDBV and EBOV, but we also isolated two antibodies that displayed potent neutralizing activity against representatives of three *Ebolavirus* species – BDBV, EBOV, and SUDV. We tested two cross-neutralizing mAbs in mice and guinea pigs and showed that they protected animals from lethal challenge with a heterologous species of EBOV. These data suggest that cross-neutralizing mAbs can be used to develop a universal treatment against multiple ebolaviruses and imply that highly immunogenic vaccines with proper presentation of GP from one species could induce some measure of cross-protection against viruses of the other species. The ability of these mAbs to bind and neutralize a broad range of *Ebolavirus* species also suggest that such antibodies might offer protection against emerging filoviruses in the future.
Figure 24. **Survival and clinical signs of EBOV inoculated guinea pigs treated with BDBV mAbs**. Groups of 5 guinea pigs per group were injected with individual mAbs by the intraperitoneal route 1 day or 1 and 3 days after EBOV challenge, using 5 mg of individual mAb (A) or 5 mg of the combination of two mAbs per treatment (B), as indicated. Animals treated with dengue virus-specific human mAb 2D22 served as controls. The survival curves are based on morning and evening observations. Mortality in the morning is shown in whole day numbers, in the evening in 1/2 day values. The body weight and illness scores are shown with one value per day.
Several antibody-based treatments provided complete species-specific protection from EBOV in non-human primate model of infection (Qiu et al., 2014). However, antibody-based therapeutics against other members of the *Ebolavirus* genus, such and BDBV and SUDV, are not available. While one strategy would be to develop separate antibody treatments for each filovirus infection, an alternative strategy would be to have a universal treatment effective against diverse *Ebolavirus* species. The development of universal antibody treatments for ebolaviruses seems inevitable, given recent progress in the identification of broad and potent neutralizing antibodies against viruses that exhibit more antigenic diversity than the filoviruses such as HIV (Burton et al., 2012), influenza viruses (Pappas et al., 2014), dengue virus (Rouvinski et al., 2015), alphaviruses (Fox et al., 2015), and paramyxoviruses (Corti et al. 2013). Our results provide a roadmap to develop a single antibody-based treatment effective against multiple *Ebolavirus* infections. I propose that the principal components of such treatment should include cross-neutralizing mAbs that target conserved elements of the non-overlapping major neutralizing antigenic sites on the GP surface.

**Materials and Methods**

**Donors**

De-identified peripheral blood mononuclear cells (PBMCs) from 7 survivors of the 2007 BDBV outbreak in Uganda (Towner et al., 2008) were obtained from a repository at Makerere University (Kampala, Uganda) managed in collaboration with the U.S. Military HIV Research Program MHRP, which is part of the Walter Reed Army Institute of Research. PBMCs were obtained after informed consent from a U.S. survivor of Ebola virus Zaire (EBOV) infection who was infected while delivering health care in Liberia during the 2014
Ebola virus outbreak with Makona virus. Cells from the EBOV survivor were obtained about 11 weeks after infection and about 7 weeks after discharge from hospital, following several negative PCR tests for presence of virus. PBMCs were obtained from a U.S. survivor of Marburg virus (MARV) infection who developed the disease in early 2008 following exposure to fruit bats in the Python Cave in Queen Elizabeth National Park, Uganda. This donor’s clinical course was documented previously (Centers for Disease and Prevention, 2009), and we have previously reported isolation of human antibodies from this donor (Flyak et al., 2015). Peripheral blood from the donor was obtained in 2012, four years after the illness, following informed consent. The studies were approved by the Vanderbilt University Institutional Review Board.

Viruses

BDBV strain 200706291 Uganda was isolated originally from the serum of a patient during the first recorded outbreak caused by this virus (Towner et al., 2008) and passaged three times in Vero E6 cells. The virus was provided originally by the Special Pathogens Branch of the U.S. Centers for Disease Control and Prevention (CDC) and deposited at the World Reference Center of Emerging Viruses and Arboviruses (WRCEVA), housed at the University of Texas Medical Branch (UTMB), Galveston, TX. The mouse-adapted EBOV strain Mayinga was originally generated by Dr. Mike Bray (U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland) (Bray et al., 1998). The virus was provided originally by the Special Pathogens Branch of CDC, deposited at WRCEVA, and amplified by one passage in Vero-E6 cells. The guinea pig-adapted EBOV strain Mayinga was generated originally by Dr. Brett Connolly (U.S. Army Medical Research Institute of Infectious Diseases) (Connolly et al., 1999) and was provided by Dr. Alexander Freiberg (UTMB) through Dr. Heinz Feldmann (Special Pathogens Program, National Microbiology Laboratory, Canadian Science Centre for Human and Animal Health,
Winnipeg, Canada) and amplified by one passage in Vero-E6 cells. The recombinant EBOV strain Mayinga expressing eGFP was generated by a reverse genetics technique (Lubaki et al., 2013) as previously described (Towner et al., 2005) from plasmids provided by Drs. Jonathan Towner and Stuart Nichol (CDC) and Drs. Yoshihiro Kawaoka (University of Wisconsin) and Heinz Feldmann (NIH), and passaged three times in Vero E6 cells. The EBOV Makona strain from the 2014-2015 West African outbreak, which was provided by Dr. T. Geisbert (UTMB), was isolated originally from serum of a fatally infected patient in early 2014 in Guekedou, Guinea, and was passaged two times in Vero E6 cells. The chimeric EBOV/BDBV-GP, EBOV/MARV-GP and EBOV/SUDV-GP constructs expressing eGFP were obtained by replacing the gene encoding EBOV GP with that of BDBV, MARV or SUDV, respectively (Ilinykh P., unpublished data), and passaged two times in Vero E6 cells.

Generation of human hybridomas secreting monoclonal antibodies (mAbs)

Human hybridomas were generated as described previously (Flyak et al., 2015). In brief, previously cryopreserved samples were transformed with Epstein-Barr virus, CpG and additional supplements. After 7 days, cells from each well of the 384-well culture plates were expanded into four 96-well culture plates using cell culture medium containing irradiated heterologous human PBMCs (recovered from blood unit leukofiltration filters, Nashville Red Cross) and incubated for an additional four days. Plates were screened for BDBV GP antigen-specific antibody-secreting cell lines using enzyme-linked immunosorbent assays (ELISAs). Cells from wells with supernates reacting with antigen in an ELISA were fused with HMMA2.5 myeloma cells using an established electrofusion technique (Yu et al., 2008).
Human mAb and Fab production and purification

After fusion, hybridoma cell lines were cloned by single-cell fluorescence-activated cell sorting and expanded in post-fusion medium as previously described (Flyak et al., 2015). HiTrap Protein G or HiTrap MabSelectSure columns were used to purify antibodies from filtered supernates. Fab fragments were generated by papain digestion, as described previously (Flyak et al., 2015).

Expression and purification of filovirus GPs

BDBV GP ectodomain (BDBV GP, residues 1-637) or the secreted glycoprotein dimer (BDBV sGP, residues 1-316) were used to screen supernatants of transformed B cells. Recombinant glycoproteins were engineered with a C-terminal double strep tag and cloned into a modified pMTpuro vector for expression in Drosophila S2 cells. Briefly, plasmids were transfected into S2 cells using Effectene reagent (Qiagen) followed by stable cell selection with 6 µg/mL puromycin. S2 cells first were cultured in Schneider’s medium supplemented with 10% (v/v) FCS (Lonza), and later adapted to Insect Xpress medium for large-scale expression in 2L shaker flasks. Stable cells were induced with 0.5 mM CuSO₄ and harvested after 4 to 5 days at 27°C. Tangential flow filtration then was used to buffer exchange the supernatants into 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 15 µg/mL avidin pH 8.0, and target proteins were purified using Streptactin Superflow affinity (Qiagen). GP ectodomains were purified further with S200 size exclusion chromatography (SEC); sGP was purified with S75 SEC. Recombinant ectodomains for EBOV, SUDV or MARV were designed and expressed similarly.

Screening and half maximal effective concentration (EC₅₀) ELISA binding analysis.

Soluble forms of the full-length extracellular domain of BDBV, EBOV, SUDV or MARV GPs or the sGP form of BDBV GP were coated overnight onto 384-well plates at 1 µg/mL. For
screening ELISA, 10 µL of supernate from a well of a tissue-culture plate were transferred to each well of a 384-well ELISA plate. For EC\textsubscript{50} binding analysis by ELISA, purified antibodies were applied to the plates at a concentration range of 30 µg/mL to 170 ng/mL, using three-fold serial dilutions. The presence of antibodies bound to the GP was determined using goat anti-human IgG alkaline phosphatase conjugate and p-nitrophenol phosphate substrate tablets, with optical density read at 405 nm after 120 minutes. A non-linear regression analysis was performed on the resulting curves using Prism version 5 (GraphPad) to calculate EC\textsubscript{50} values. The Circos software package was used for data visualization (Krzywinski et al., 2009).

**EBOV and MARV neutralization experiments**

Isolated mAbs were screened initially in a high-throughput neutralization assay using EBOV/BDBV-GP with or without 5% guinea pig complement (MP Biomedicals, Santa Ana, CA) (Illynykh P., unpublished data). The mAbs that exhibited neutralizing activity also were screened for neutralization of eGFP-expressing EBOV (Towner et al., 2005). BDBV223 was tested for neutralization of EBOV/SUDV-GP and EBOV/MARV-GP by the same approach. In addition, neutralizing activity of BDBV223 was tested against the EBOV Makona strain by a classic plaque reduction assay, which was performed as follows. Triplicate samples of 150 PFU of the virus were mixed with serial dilutions of mAbs, with or without 5% guinea pig complement in a total volume of 100 µL, incubated for 1 hour at 37°C, and placed on Vero E6 cell monolayers. After a 1 hour-long virus adsorption at 37°C, cells were overlaid with 0.8% tragacanth (Spectrum Chemical Mfg. Corp., New Brunswick, NJ) solution in Minimal Essential Medium containing 10% FBS (HyClone, Logan, Utah) and 0.1% gentamicin (Mediatech, Manassas, VA), and incubated for 14 days. Plaques were visualized by staining of monolayers with 0.25% crystal violet (Thermo Fisher Scientific, Waltham, MA) in 10% formalin.
Biolayer interferometry competition binding assay

Competition binding studies using biolayer interferometry and biotinylated BDBV GP (EZ-link® Micro NHS-PEG₄-Biotinylation Kit, Thermo Scientific #21955) (5 µg/mL) were performed on an Octet RED biosensor (ForteBio, Menlo Park, CA), as described previously (Flyak et al., 2015). In brief, the antigen was immobilized onto streptavidin-coated biosensor tips. After a brief washing step, biosensor tips were immersed first into the wells containing first antibody at a concentration of 100 µg/mL and then into the wells containing a second mAb at a concentration of 100 µg/mL. The percent binding of the second mAb in the presence of first mAb was determined by comparing the maximal signal of the second mAb applied after the first mAb complex to the maximal signal of the second mAb alone.

Sequence analysis of antibody variable region genes

Antibody variable gene sequence analysis was performed as previously described (Flyak et al., 2015). Heavy chain antibody variable region sequences were analyzed using the IMGT/V-Quest program (Brochet et al., 2008; Giudicelli et al., 2011).

Electron microscopy and sample preparation

Fabs were added in 10 molar excess to BDBV GPdMuc and subsequently purified and stained as previously described (Murin et al., 2014).

Image processing of protein complexes

Particles were automatically picked using DoG Picker (Voss et al., 2009) and particle stacks were generated using Appion (Lander et al., 2009). Subsequently, reference-free 2D class averages were generated using iterative MRA/MSA (van Heel et al., 1996). Non-GP complexes and those with a clear lack of full saturation by Fab were removed to generate a
final stack for reconstructions. In some cases, orientation bias or flexibility of Fabs prevented convergence of an acceptable model, although examination of class averages allowed a general assignment of the epitope. Final stack class averages were used to generate initial models using EMAN2 common lines (Tang et al., 2007). A model matching its reference projections was further refined using the entire raw particle stack with EMAN2, as described previously (Murin et al., 2014). For the BDBV41 reconstruction, the EMAN2 reconstruction lacked important features that were present in the class averages, indicating that perhaps some particles lacked full Fab saturation. In order to circumvent this problem, we utilized the Relion package, which allows 3D-classification to remove particles that may only contain 2 Fabs, significantly improving the quality of the final EM map (Scheres, 2012). Modeling fitting and EM figures were generated using UCSF Chimera (Pettersen et al., 2004).

Epitope mapping using an EBOV GP alanine-scan mutation library

Comprehensive high-throughput alanine scanning (‘shotgun mutagenesis’) was carried out on an expression construct for EBOV GP (Yambuku-Mayinga variant GP; Uniprot accession number Q05320). Residues 33-676 of full-length EBOV GP were mutagenized to create a library of clones, each representing an individual point mutant. Residues were changed to alanine (with alanine residues changed to serine). GP residues 1-32, which constitute the GP signal peptide, were not mutagenized. The resulting EBOV GP alanine-scan library covered 99.5% of target residues (641 of 644). Clones were arrayed into 384-well plates, one mutant per well. The EBOV GP mutation library was transfected into HEK-293T cells and allowed to express for 22 hours. Cells were fixed in 4% paraformaldehyde in PBS plus calcium and magnesium, or were left unfixed, and were then incubated with an Ab diluted in 10% normal goat serum (NGS) (Sigma-Aldrich, St. Louis, MO). The cells were incubated with primary antibody for 1 hour at room temperature, followed by a 30 minute incubation with Alexa Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch...
Laboratories, Westgrove, PA) in 10% NGS. Cells were washed twice with PBS without calcium or magnesium and resuspended in Cellstripper (Cellgro, Manassas, VA) plus 0.1% BSA (Sigma-Aldrich, St. Louis, MO). Cellular fluorescence was detected using the Intellicyt high throughput flow cytometer (Intellicyt, Albuquerque, NM). Background fluorescence was determined by fluorescence measurement of vector-transfected control cells. Ab reactivities against each mutant EBOV GP clone were calculated relative to wild-type EBOV GP reactivity by subtracting the signal from mock-transfected controls and normalizing to the signal from wild-type GP-transfected controls.

Before screening, the immunoreactivities of MAbs BDBV270, BDBV289, and BDBV324 were optimized by determining reactivity with fixed or unfixed cells over a range of mAb concentrations to identify optimal signal-to-background ratios (>5:1) and to ensure that signals were within the linear range of detection. MAb BDBV289 also screened as a Fab after conversion by papain digestion. Control mAbs 2G4 and 4G7 were kindly provided by Gary Kobinger, Public Health Agency of Canada.

Mutated residues within critical clones were identified as critical to the Ab epitope if they did not support reactivity of the test Ab but did support reactivity of other control EBOV mAbs. This counter-screen strategy facilitates the exclusion of GP mutants that are locally misfolded or that have an expression defect. The detailed algorithms used to interpret shotgun mutagenesis data are described elsewhere (patent application 61/938,894), (Davidson and Doranz, 2014).

**Generation and Sequence Analysis of VSV/BDBV GP Escape Mutants**

Briefly, 200 PFUs of VSV/BDBV-GP virus were pre-incubated with 2-fold decreasing concentrations of mAbs before each passage, starting from 200 µg/mL, and serially
passaged 3-10 times under selective pressure of the corresponding mAbs. After each passage, virus aliquots were harvested and titrated. A suspension containing 200 PFUs from the virus-positive aliquot with the highest mAb concentration was used for the next passage. Finally, viruses were plaque-purified, and the genes encoding the BDBV GPs were sequenced. Viral samples derived from plaques containing any amino acid substitutions were propagated further in the presence of the corresponding mAb, and tested for neutralization resistance by plaque reduction assay.

In vivo testing

The animal protocols for testing of mAbs in mice and guinea pigs were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston. Seven-week-old BALB/c mice (Charles River Laboratories) were placed in the ABSL-4 facility of the Galveston National Laboratory. Groups of mice at 5 animals per group were injected with 1,000 PFU of the mouse-adapted EBOV by the intraperitoneal route. Twenty-four or seventy four hours later, animals were injected with individual mAbs by the intraperitoneal route using 100 µg per treatment. Animals treated with the antibody specific to dengue virus 2D22 served as controls. Animals were weighed and monitored daily over the two-week period after challenge. Once animals were symptomatic, they were examined no less than twice per day. The disease was scored using the following parameters: dyspnea (possible scores 0-5), recumbency (0-9), unresponsiveness (0-5), and bleeding/hemorrhage (0-5). To test the protective efficacy of mAbs in guinea pigs, five to six week-old animals (strain Hartley) were placed in the ABSL-4 facility of the Galveston National Laboratory. Groups of 5 animals per group were injected with 1,000 PFU of guinea pig-adapted EBOV, by the intraperitoneal route. Twenty-four hours later and 72 hours later, animals were injected with individual mAbs (5 mg per treatment), or a cocktail of two mAbs (2.5 mg of each mAb per treatment). Animals were weighted and monitored daily for 14
days. After animals became symptomatic, they were examined no less than twice per day. The disease was scored using the following parameters: appearance (possible scores 0-3), body condition (0-3), natural behavior (0-3), and provoked behavior (0-3).
CHAPTER IV

CROSS-REACTIVE HUMAN ANTIBODIES TO THE HR2/MPER REGION OF EBOLA GLYCOPROTEIN

“...the warriors against Ebola understand that they face a long struggle against a formidable enemy. Many of their weapons will fail, but some will begin to work”

Richard Preston, Inside the Ebola Wars, The New Yorker

Introduction

The antibody cocktail ZMapp™ is effective in nonhuman primate models of infection (Qiu et al., 2014) and has been used under compassionate-treatment protocols in humans (Lyon et al., 2014). ZMapp™ is a mixture of three humanized murine mAbs (Olinger et al., 2012b; Qiu et al., 2011; Qiu et al., 2012b; Wilson et al., 2000) that target EBOV-specific epitopes on the surface GP (Davidson et al., 2015; Murin et al., 2014). As a result, ZMapp™ mAbs do not neutralize other viruses from the Ebolavirus genus, such as BDBV or SUDV.

In this chapter, I describe three naturally-occurring human cross-reactive mAbs from BDBV survivors that target a new antigenic site in the canonical heptad repeat 2 (HR2) region near the membrane proximal region of EBOV GP. I found that cross-reactive HR2/MPER-specific antibodies do not compete with previously isolated EBOV-specific mAbs that recognize other regions in the GP, such as the glycan cap, base, or mucin-like domain, but rather they bind a site near the viral membrane. The identification of a conserved protective antigenic site in the GP suggests that these mAbs can be used to design antibody therapeutics against multiple filovirus infection. Furthermore, structural
features formed by conserved residues in the protective site could be used to develop an epitope-based vaccine against infection caused by diverse *Ebolavirus* species.

I acknowledge Alexander Bukreyev’s group for performing neutralization, protection experiments as well as escape mutants generation, Andrew Ward’s group for studying EBOV-specific mAbs by negative-stain single-particle EM, Erica Saphire’s group for providing *Ebolavirus* GPs, Ben Doranz’s team for performing epitope mapping of selected mAbs using saturation mutagenesis, and David Wright’s group for peptide synthesis.

Cross-reactive neutralizing antibodies bind a unique region on GP surface

In Chapter III, I described the isolation of a large panel of neutralizing mAbs using peripheral B cells from survivors of the 2007 BDBV outbreak in Uganda. Among seven cross-reactive mAbs that bound to GPs from multiple ebolaviruses (BDBV, EBOV, and SUDV), I found three neutralizing mAbs (designated BDBV223, BDBV317, and BDBV340) that bound to the recombinant trimeric form of GP ectodomain but did not bind to the dimeric form of GP that is secreted from cells during infection (sGP, secreted GP) (*Figure 18A, 19A*). As BDBV223, BDBV317, and BDBV340 recognized all three GPs from BDBV, EBOV, and SUDV (*Figure 18A, Group 3B*), we next sought to determine whether these cross-reactive mAbs could neutralize viruses from those three *Ebolavirus* species. All three mAbs neutralized autologous BDBV as well as heterologous EBOV. Although these human mAbs recognized SUDV GP in ELISA, BDBV223, BDBV317, and BDBV340 failed to neutralize SUDV (*Figure 25A*). Among three mAbs, BDBV223 displayed an extremely high neutralizing potency, with BDBV IC$_{50}$ values – 0.01 ng/mL and EBOV IC$_{50}$ values – 1.8 µg/mL (*Figure 25A*).
To determine the therapeutic activity of these cross-neutralizing Abs, we tested these three antibodies in mice. Seven week-old BALB/c mice received 100 µg of antibody by the IP route 1 day after inoculation with 1,000 PFU of mouse-adapted EBOV, strain Mayinga (Bray et al., 1998). Both BDBV223 and BDBV317 fully protected mice from lethal challenge with heterologous species of EBOV (Figure 25B). We did not observe a protective effect in mice receiving treatment with BDBV340 or dengue-specific antibody 2D22 (Figure 25B).

There are several neutralizing mAbs that bind to the base region of the filovirus GP, including two chimerized murin-origin antibodies from the ZMap™ cocktail (c2G4 and c4G7) (Qiu et al., 2011; Qiu et al., 2012b), the human phage-display library-derived mAb KZ52 (Maruyama et al., 1999), and the murine SUDV-specific mAb 16F6 (Dias et al., 2011a). The epitopes bound by these mAbs were defined crystallographically (KZ52 and 16F6) (Dias et al., 2011a; Lee et al., 2008a), by negative-stain single-particle EM (c2G4 and
To determine whether BDBV223, BDBV317, and BDBV340 compete for binding with these *Ebolavirus*-specific mAbs, I performed quantitative competition-binding assay using an Octet biosensor (Figure 26). I also included in this analysis a previously isolated potently neutralizing human mAb (BDBV289) that recognizes the glycan cap (Figure 23) as well as...
the glycan cap-specific mAb c13C6 from the ZMapp™ cocktail (Wilson et al., 2000). As expected, the glycan cap-specific mAbs (c13C6 and BDBV289) and the base region-binding mAbs (KZ52, c2G4, and c4G7) segregated into two independent competition-binding groups (Figure 26). BDBV223, BDBV317, and BDBV340 did not compete with either glycan-cap specific mAbs or base region-binding mAbs, suggesting that these new mAbs target a unique antigenic region on the GP surface (Figure 26).

Electron microscopy studies of cross-reactive mAbs from Group 3B

To determine the location of the cross-reactive antigenic site on the GP base targeted by these human mAbs, we performed negative-stain single-particle EM studies in collaboration with Andrew Ward’s group. The EM class averages clearly showed that BDBV223, BDBV317, and BDBV340 each bind to the bottom of GP, in the canonical heptad repeat 2 (HR2) domain near the membrane proximal external region (MPER) (Figure 27A). Overlaying a class average of BDBV223 Fab bound to BDBV GP (Figure 27B) over a class average of c13C6 Fab:c4G7 Fab bound to EBOV GP (Figure 27C) showed that BDBV223, and the two other new mAbs bound more virion proximal on the GP, well below the epitope of the mAb c4G7 site of vulnerability at the GP1/GP2 interface (Figure 27D). Measurements of the distance from the bottom of the GP globular head to the mid-point of the Fab in the class averages showed a distance of ~60 Å, which corresponds to the length of the HR2 region previously crystalized as post-fusion GP2 (PDBID 1EBO) (Figure 27E). Three BDBV317 Fabs can be seen in the class average images, indicating that three mAbs can bind simultaneously to the HR2/MPER region, which lies in a close proximity to the viral membrane (Figure 27A). In Chapter III, I described species-specific neutralizing human
mAbs that bound the BDBV GP near the MPER region and competed for binding with cross-reactive mAbs BDBV223, BDBV317, and BDBV340, although their epitopes were not determined (Figure 21, 22). Also, a previous study described generation of a murine cross-reactive non-neutralizing mAb to the HR2/MPER region (15H10) (Yu et al., 2006). Therefore, HR2 and MPER regions are complex major antigenic sites containing species-specific, cross-reactive as well as neutralizing and non-neutralizing epitopes.

Analysis of GP residues required for mAb cross-reactivity and neutralization

To define the epitope of cross-reactive human mAbs more precisely, we collaborated with Ben Doranz’s group at Integral Molecular and tested the binding of BDBV223, BDBV317, and BDBV340 or the ZMapp™ antibody c4G7 to individual GP members of an
alanine-scan mutant library of EBOV GP. Several critical residues were identified previously for c4G7, including D552 in the internal fusion loop and C556, which is part of the HR1 (Davidson et al., 2015). Consistent with the EM data, we identified critical residues in GP2 for BDBV223, BDBV317, and BDBV340 that spanned the C-terminal part of HR2 and N-terminal part of the MPER (Figure 28A). A single critical residue in HR2 was identified for BDBV340 (D624), and one residue in MPER was identified for BDBV317 (K633). Two critical residues were identified for BDBV223 (D624 and D632) (Figure 28A).

The HR2 α-helix ends at residue D632 (Malashkevich et al., 1999; Weissenhorn et al., 1998), indicating that HR2/MPER peptides might adopt an α-helical conformation that is recognized by the Ebolavirus cross-reactive mAbs. To determine whether BDBV223, BDBV317, or BDB340 can recognize a linear conserved epitope in HR2/MPER, we synthesized peptides spanning the GP amino acids 620-635 in the HR2/MPER of three virus species (designated peptides BDBV2, EBOV2, and SUDV2) as well as a control peptide from the N-terminus of HR2 (amino acids 599-613), designated the BDBV1 peptide. I determined that BDBV223, BDBV317, and BDBV340 recognize BDBV2 peptide containing the essential residues determined by alanine-scanning mutagenesis but not the negative control BDBV1 peptide from HR2, as expected (Figure 28B). While BDBV223 bound to BDBV2, EBOV2, and SUDV2 peptides, BDBV317 and BDBV340 recognized only BDBV2 and EBOV2 peptides (Figure 28B). Interestingly, I noticed similarities between the extent of binding of the three mAbs to the HR2/MPER peptides (Figure 28B) and their neutralization potencies in vitro (Figure 25B). BDBV317 bound equally well to BDBV2 and EBOV2 peptides and neutralized BDBV and EBOV with the same potency. BDBV223 and BDBV340 bound better to BDBV2 peptide, and the same antibodies neutralized BDBV with higher potency than EBOV (Figure 25B).
To determine the basis of mAb cross-reactivity to multiple Ebolavirus GPs and peptides, we synthesized a panel of chimeric BDBV peptides containing polymorphic residues from EBOV or SUDV. There are two amino acid differences between BDBV2 and EBOV2 peptides (at residues 631 and 634), located in the C-terminus of the peptide (Figure 28).
To identify polymorphisms responsible for the enhanced binding of BDBV223 to BDBV2 peptide, we synthesized two chimeric BDBV peptides with single substitutions at residues 631 or 634 (designated peptides BDBV2A and BDBV2B). While BDBV223 bound equally well to BDBV2 and BDBV2A peptide, it bound relatively weakly to the BDBV2B peptide, suggesting that the P634T substitution in BDBV2B peptide is responsible for reduced binding of BDBV223 to the EBOV2 peptide (Figure 28C). We also synthesized chimeric BDBV2C and BDBV2D peptides to introduce two SUDV substitutions at residues 624 and 633. While both BDBV223 and BDBV317 bound equally well to BDBV2D and BDBV2 peptides, they failed to recognize the BDBV2C peptide, suggesting that the N624 residue is required for neutralization of SUDV by MPER/HR-specific mAbs. To investigate the role residue D624 in the context of a live filovirus, we constructed a recombinant BDBV bearing D624N mutation, which was found to be completely resistant to BDBV223 but sensitive to BDBV317 (Figure 29A).

We passaged the chimeric filovirus with BDBV GP (Ilinykh et al., 2016) in the presence of HR2/MPER-specific mAbs and generated antibody escape mutant viruses for mAbs BDBV223 and BDBV317. For BDBV223, an isolate with a P634H mutation in the MPER was identified, and for BDBV317 an isolate with a K633R mutation in the MPER was identified (Figure 29B). We found that while the BDBV223 escape mutant was resistant to neutralization by both BDBV223 and BDBV317, BDBV223 was able to neutralize the BDBV317 escape mutant (Figure 29B). This finding is consistent with the results from the alanine-scanning and peptide ELISA binding experiments, in which the K633 residue was identified as a critical residue only for BDBV317 (Figure 28A). Also, BDBV317, but not BDBV223, failed to bind in ELISA to the BDBV3D peptide with a K633N substitution (Figure 28C).
Discussion

In this chapter, I describe three human cross-reactive antibodies from Group 3B that target a new antigenic region near the MPER of EBOV GP. These antibodies do not compete with therapeutic antibodies used in the past to treat EBOV infection, suggesting that these MPER-specific mAbs could be used to design a new universal antibody therapeutic cocktail against multiple species of viruses causing Ebolavirus infection. Alternatively, the mAbs might be beneficial to include in existing experimental therapeutic antibody cocktails to increase the potency and breadth of those combinations. Although these mAbs are the first reported neutralizing antibodies directed to the MPER of filoviruses, the MPER in the GP of enveloped viruses increasingly is recognized as an important region for recognition by broad and potent human mAbs. Several mAbs that neutralize a broad
range of HIV strains bind conserved epitopes in the HIV gp41 MPER (Muster et al., 1993; Zwick et al., 2001). The identification of potent HIV MPER-specific mAbs has facilitated important progress in the effort to design HIV vaccines rationally using antigens designed to induce such mAbs (Montero et al., 2008). Neutralizing mAbs that recognize the influenza hemagglutinin surface protein stem region (Corti et al., 2011; Ekiert et al., 2009; Sui et al., 2009) also have simulated research into the possibility of a universal influenza vaccine. Here, I report structural and functional information about conserved epitopes in the MPER of EBOV GP that can be used to inform the development of an MPER-based EBOV vaccine effective against multiple filoviruses.

Materials and Methods

Human mAb and Fab expression and purification

Human hybridoma cell lines were expanded in post-fusion medium, as previously described (Flyak et al., 2015). HiTrap Protein G or HiTrap MabSelectSure columns were used to purify antibodies from filtered supernates. Fab fragments were generated by papain digestion, as described previously (Flyak et al., 2015).

Expression and purification of filovirus GPs

Recombinant GP ectodomains containing the mucin-like domain (GPΔTM) or lacking residues 312–463 of the mucin-like domain (GPΔmuc) (Lee et al., 2009; Lee et al., 2008b) were produced by transfection of Drosophila Schneider 2 (S2) cells with modified pMTpuro vectors, followed by stable selection of transfected cells with 6 µg/mL puromycin. Secreted GP ectodomain expression was induced with 0.5 mM CuSO4 for 4 d. Proteins were engineered with a modified double strep tag at the C terminus (enterokinase cleavage site
followed by a strep tag/linker/strep tag) to facilitate purification using Strep-Tactin resin (Qiagen). Proteins were purified further by Superdex 200 (S200) SEC in 10 mM Tris and 150 mM NaCl, pH 7.5 (1× TBS).

Screening and half maximal effective concentration \((EC_{50})\) ELISA binding analysis

Soluble forms of the full-length extracellular domain of BDBV, EBOV, SUDV or MARV GPs or the sGP forms were coated overnight onto 384-well plates at 1 µg/mL. For screening ELISA, 10 µL of supernate from a well of a tissue-culture plate were transferred to each well of a 384-well ELISA plate. For \(EC_{50}\) binding analysis by ELISA, purified antibodies were applied to the plates at a concentration range of 30 µg/mL to 170 ng/mL, using three-fold serial dilutions. The presence of antibodies bound to the GP was determined using goat anti-human IgG alkaline phosphatase conjugate and \(p\)-nitrophenol phosphate substrate tablets, with optical density read at 405 nm after 120 minutes. A non-linear regression analysis was performed on the resulting curves using Prism version 5 (GraphPad) to calculate \(EC_{50}\) values.

Antibody neutralization experiments

All work with filoviruses, including the chimeric filoviruses, was performed in the BSL-4 facility of the Galveston National Laboratory. Antibody neutralization assays were performed against the recombinant EBOV expressing green fluorescent protein from an added gene (Towner et al., 2005) and its derivatives in which GP was replaced with its counterpart from BDBV (strain 200706291 Uganda) or SUDV (strain 200011676 Gulu) (Ilinykh et al., 2016) to which we refer in Results as EBOV, BDBV and SUDV for simplicity. The assays were performed in a high-throughput format, as previously described (Ilinykh et al., 2016).
Generation of recombinant BDBV with the D624N mutation

To introduce the D624N mutation in BDBV GP of the chimeric filovirus used in the study (Ilinykh et al., 2016) the pEBOwtΔBamHI-SbfI,Ascl-PspOMI subclone of the full-length clone encoding the viral genome(Ilinykh et al., 2016) was mutagenized using the QuikChange site-directed mutagenesis kit (Stratagene). Then the ApaI-Sacl fragment of the subclone, which includes the mutated GP, was used to replace the corresponding fragment of the chimeric filovirus full-length clone. The mutagenized chimeric virus was recovered as previously as described (Lubaki et al., 2013).

Generation and testing of antibody escape filovirus mutants

To generate escape mutants, 100 PFU of recombinant chimeric EBOV with GP derived from BDBV were combined with 2-fold dilutions of mAbs starting at 200 µg/mL in U-bottom 96-well plates and incubated for 1 hr at 37°C. Mixtures were placed on Vero-E6 cell monolayer cultures in 96-well plates and incubated for 1 hr. Supernatants were removed, fresh mAbs were added at the same concentrations in 200 µL of MEM supplemented with 2% FBS, and plates were incubated for 7 days at 37°C. Viruses that replicated in the presence of the highest concentrations of mAbs, as determined by UV microscopy, were collected. 20 µL aliquots were incubated with 2-fold dilutions of mAbs starting at 200 µg/mL, and viruses were propagated in the presence of mAbs as above. The procedure was repeated once more with mAb dilutions starting at 400 µg/mL. Viruses that replicated at the highest mAb concentrations were amplified in Vero-E6 cell culture monolayers in 24-well plates in the presence of mAbs at 200 µg/mL for 7 days. Cells were used for isolation of RNA using TRIzol reagent, and GP genes were PCR-amplified and sequenced. To determine susceptibility of the isolated escape mutants to mAbs, 100 PFU of the viruses in MEM supplemented with 2% FBS in triplicate were combined in U-bottom 96-well plates with 8 to 12 two-fold dilutions of mAbs, staring at 200 µg/mL, in total volumes of 50 µL, and
incubated for 1 hr at 37°C. The virus/antibody mixtures then were placed in triplicate Vero-E6 cell culture monolayers in 96-well plates, incubated for 1 hr at 37°C, washed with MEM, overlaid with 200 µL of MEM containing 2% FBS and 0.8% methylcellulose, and incubated for 48 hrs at 37°C. Plates were fixed with 10% phosphate-buffered formalin (Fisher) and taken out of the BSL-4 facility according the UTMB BSL-4 standard operating procedures. Plaques were counted using a fluorescence microscope.

**Biolayer interferometry competition binding assay**

Competition-binding studies using biolayer interferometry and biotinylated EBOV GP (EZ-link® Micro NHS-PEG₄-Biotinylation Kit, Thermo Scientific #21955) (5 µg/mL) were performed on an Octet RED biosensor (ForteBio Menlo Park, CA), as described previously (Flyak et al., 2015). In brief, the antigen was immobilized onto streptavidin-coated biosensor tips. After a brief washing step, biosensor tips were immersed first into the wells containing primary antibody at a concentration of 100 µg/mL and then into the wells containing competing mAbs at a concentration of 100 µg/mL. The percent binding of the competing mAb in the presence of the first mAb was determined by comparing the maximal signal of competing mAb applied after the first mAb complex to the maximal signal of competing mAb alone.

**Electron microscopy**

To determine the epitope of HR2/MPER-directed mAbs, BDBV223, 317, or 340 Fabs were generated as described above and added in 10M excess to BDBV GPΔmuc and allowed to bind overnight at 4°C (Flyak et al., 2016). Complexes were subsequently purified by size exclusion chromatography on an S200 Increase column (GE) and stained as previously described (Murin et al., 2014). Particles were visualized using an FEI Tecnai
Spirit electron microscope operating at 120kV and images were collected on a TVIPS TemCam-F416 (4k x 4k) CCD camera using Leginon (Suloway et al., 2005) with the following settings: magnification of 52,000X that resulted in a pixel size of 2.05Å at the specimen plane, a constant defocus of -1.00 µm and an electron dosage of ~30e/Å². Images were processed using the Appion platform (Lander et al., 2009). Particles were picked using DoG Picker (Voss et al., 2009) stacks were created and 2D reference-free class averages were generated using iterative MRA-MSA (van Heel et al., 1996). For all complexes, there was a strong bias toward side-views. Further, the region containing the HR2/MPER epitope in our soluble GP constructs is flexible, as indicated by the variety of positions that bound Fabs adopted in the class averages. Therefore, our data was refractory to a reconstruction, although class averages could be compared to previous class averages of known complexes to determine the spatial location of the epitope on GP.

Epitope mapping using an EBOV GP alanine-scan mutation library

Epitope mapping was carried out as described previously. Comprehensive high-throughput alanine scanning (‘shotgun mutagenesis’) was carried out on a full-length EBOV GP expression construct (based on the Yambuku-Mayinga variant GP sequence), mutagenizing GP residues 33-676 to create a library of clones, each representing an individual point mutant. Residues were changed to alanine (with alanine residues changed to serine). The resulting library, covering 641 of 644 (99.5%) of target residues was arrayed into 384-well plates, one mutant per well, then transfected into HEK-293T cells and allowed to express for 22 hours. Cells, unfixed or fixed in 4% paraformaldehyde, were incubated with primary antibody then with an Alexa Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Westgrove, PA). After washing, cellular fluorescence was detected using the Intellicyt high throughput flow cytometer (Intellicyt, Albuquerque, NM). MAb reactivity against each mutant EBOV GP clone was calculated relative to wild-type
EBOV GP reactivity by subtracting the signal from mock-transfected controls and normalizing to the signal from wild-type GP-transfected controls.

Mutated residues within clones were identified as critical to the MAb epitope if they did not support reactivity of the test mAb but did support reactivity of other control EBOV mAbs. This counter-screen strategy facilitated the exclusion of GP mutants that were misfolded locally or that exhibited an expression defect. The detailed algorithms used to interpret shotgun mutagenesis data were described previously (Davidson and Doranz, 2014)

Peptide synthesis and purification

Peptides were synthesized using standard Fmoc solid-phase methods on a Peptide Machines Discovery-4 synthesizer on rink resin (Atherton, 1988; Bodanszky and Bodanszky, 1994; Fields and Noble, 1990; Grant, 1992; Stuber et al., 1989). All coupling reactions were performed with ten-fold excess (vs. load capacity of the resin) of activated amino acid (Aapptec or Advanced Chemtech), using FMOC amino acids/HBTU/HoBt/DIEA (1:1:1:2.5) in DMF for 60 minutes. Deprotection of the FMOC group was accomplished in 20% v/v piperidine diluted in DMF for 30 minutes. Peptides used for ELISA experiments were acetylated at the N-terminus by reaction with 1:1:2 v/v acetic anhydride:DIEA:DMF. Peptides used for biolayer interferometry assays were functionalized with PEG₆-biotin (Quanta BioDesigns) at the N-terminus.

The peptides were cleaved by exposure to a 90:5:3:2 v/v mixture of trifluoroacetic acid, thioanisole, ethanedithiol, and anisole for two hours. Peptides were precipitated by addition of cold diethyl ether, lyophilized and purified by reverse-phase HPLC (Waters Prep LC 4000) equipped with a Waters 2487 detector and C18 column. Final purified fractions were lyophilized until further use. Isolation of the target peptide was confirmed by MALDI mass spectrometry.
CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

“If Ebola can change, we can change, too, and maybe faster than Ebola”

Richard Preston, *Inside the Ebola Wars, The New Yorker*

Thesis summary

Filoviruses cause a highly lethal disease in humans, with untreated mortality rates approaching 90%. Multiple experimental strategies are being investigated for treatment of *Ebolavirus* infection. Among these drug candidates, antibody combinations exceed the efficacy and treatment window of other experimental therapeutics described so far (Qiu et al., 2014). The key components of such therapeutic cocktails contain antibodies that bind to neutralizing epitopes on the GP surface. Most of our knowledge about neutralizing antibody responses against filovirus infections has come from studies of murine mAbs that recognize EBOV GP. As a result, there is little information available about human antibody responses to EBOV infection, and we have very limited knowledge about neutralizing antibodies against other filoviruses, such as BDBV, SUDV and MARV.

I began studies of human antibody responses to filovirus infection by generating a large panel of MARV-specific human mAbs from B cells of an individual who contracted MARV infection in 2008 following exposure to fruit bats in the Python Cave in Queen Elizabeth National Park in Uganda (Chapter II). Among the 39 MARV GP-specific mAbs, we found 18 mAbs that exhibited neutralization activity against chimeric vesicular stomatitis virus with MARV GP from the Uganda strain on its surface (VSV/GP-Uganda). All VSV/GP-Uganda neutralizing Abs displayed unique binding patterns in ELISA and blocked the
binding of each of the other neutralizing Abs to the antigen in an Octet competition experiment. These data suggested that MARV-specific neutralizing Abs bind a single antigenic region on the GP surface.

To determine the location of the antigenic region targeted by MARV neutralizing Abs, we performed negative stain single-particle EM studies using antibody-GP complexes. We observed that all of the neutralizing antibodies bind to MARV GP at or near the predicted region of the NPC1 receptor-binding site. As all neutralizing Abs segregated into a single competition group and bound the MARV GP at the NPC1 receptor-binding site, I proposed that blocking of MARV GP binding to NPC1 is the principal mechanism of MARV neutralization by naturally-occurring human Abs.

To determine whether MARV-specific human Abs could also bind in a cross-reactive manner to the EBOV GP receptor-binding site, I performed ELISAs using EBOV GP. I found that three of the MARV neutralizing Abs recognized the EBOV GPcl that lacked the glycan cap and mucin-like domain. However, structural analysis of MARV and EBOV GPs revealed that the glycan cap and mucin-like domain likely obscure the receptor-binding domain in EBOV. In agreement with the structural data, MARV neutralizing Abs did not exhibit detectable neutralization activity against EBOV or VSV/EBOV.

While I was not able to generate pan-filovirus neutralizing Abs using B cells from a MARV survivor, I decided to test the idea that functionally important regions of GP can be conserved between multiple species from the *Ebolavirus* genus. The serum of individuals who survived BDBV, EBOV, or SUDV infections contained *Ebolavirus* cross-reactive antibodies, suggesting that conserved neutralizing epitopes can be found on the surface of *Ebolavirus* GP. In Chapter III, I describe the isolation and characterization of a large panel of human antibodies using B cells obtained from survivors of a BDBV outbreak.
I found that 57 of 90 mAbs generated from B cells of BDBV survivors recognized heterologous EBOV GPs. Some of the isolated cross-reactive mAbs also neutralized multiple *Ebolavirus* species. The majority of cross-reactive mAbs neutralized BDBV and EBOV, but I also isolated two antibodies that displayed neutralizing activity against representatives of three *Ebolavirus* species – BDBV, EBOV, and SUDV. We tested two cross-neutralizing mAbs in mice and guinea pigs and showed that they protected animals from lethal challenge with a heterologous species of EBOV. We demonstrate that glycan cap-specific mAbs exhibit very potent neutralizing activity, and they recognize diverse epitopes within this major antigenic region.

In Chapter IV, I describe three cross-reactive mAbs isolated from BDBV survivors that bind to a new antigenic site in the HR2 region near the EBOV GP MPER. I found that these cross-reactive HR2/MPER-specific antibodies do not compete with previously isolated EBOV-specific mAbs that recognize other regions in the GP, such as the glycan cap, base, or mucin-like domain. We used a series of protein, virologic, and structural biology studies to define the GP residues important for HR2/MPER mAb cross-reactivity and neutralization.

Collectively, findings presented in this thesis enhance our understanding of human antibody responses to filovirus infection. The new knowledge gained from this study could help develop broad-spectrum protective mAbs and detection capabilities for existing filoviruses, and new viral strains that may emerge in the future. Information gathered from this thesis reveals mechanisms of filovirus neutralization by human mAbs and provides information about conserved epitopes on GP that can be used to inform the development of vaccines effective against multiple filoviruses.
Future directions

Bispecific antibodies as pan-filovirus therapeutic agents

In Chapter II, I described a large panel of MARV neutralizing mAbs isolated from B cells of an individual who contracted MARV infection. Among MARV-specific neutralizing Abs, I found several that recognize the EBOV receptor-binding site (Figure 15A), suggesting that the binding site for the essential intracellular receptor NPC1 could be an attractive target for broadly neutralizing Abs. However, the glycan cap and mucin-like domain obscure the receptor-binding site in EBOV GP, complicating the development of pan-filovirus Ab treatment. Protein engineering approaches could be employed to deliver neutralizing Abs that bind to the filovirus receptor-binding site to late endosomes or lysosomes, where the NPC1-binding site is unmasked by host proteases.

Bispecific Abs (bsAbs) combine specificities of two Abs and can simultaneously bind to different antigens or epitopes. The bsAbs have two heavy and two light chains, one each from two different Abs. The two Fab regions are directed against two antigens or epitopes. While the generation of bsAbs in a practical and efficient manner has been a longstanding challenge, platforms have been developed that improve bsAb product homogeneity and yield (Labrijn et al., 2014; Lewis et al., 2014). For example, in a Fab-arm exchange method, two parental Abs containing single matching point mutations in the CH3 domain are mixed under permissive redox conditions to enable recombination of half-molecules (Figure 30). This method could be employed to generate pan-filovirus bsAbs. For this approach, a bsAb could be designed to contain variable domains for a human neutralizing Ab that binds to the conserved NPC1 receptor-binding site and an Ebolavirus cross-reactive Ab that binds to the glycan cap.
During the *Ebolavirus* infection, the cross-reactive glycan cap-specific Fab-fragment would bind to conserved epitopes on EBOV GP surface and deliver neutralizing Fab-fragment that binds to the receptor-binding site to endosomes where EBOV NPC1 receptor-binding site is exposed for Ab binding. Such a bsAb should display neutralizing activity against multiple MARV and EBOV species. Neutralization and protection experiments could be performed to test the inhibitory and protective effect of such bsAb.

**Mechanisms of *Ebolavirus* neutralization by glycan cap-specific mAbs**

In Chapter III, I highlight the neutralization and protective potencies of human glycan cap-specific antibodies. Glycan cap-binding mAbs might not neutralize well because host cathepsins remove this region during viral entry (Murin et al., 2014). However, several of the BDBV glycan cap-specific mAbs described in Chapter III exhibit very potent neutralizing activity and recognize diverse epitopes within this major antigenic site. Furthermore, a single glycan cap-specific neutralizing Ab, BDBV289, provides complete protection in EBOV-challenged guinea pigs. The mechanism used by glycan cap-binding mAbs to neutralize the
virus in vitro is unclear. While the amino acid sequence of the GP1 region is generally less well conserved than that of GP2 in viruses of diverse filovirus species, the five neutralizing glycan cap mAbs described in Chapter III target conserved residues, suggesting that these regions are important to the viral lifecycle. Therefore, these mAbs may inhibit some yet undefined function of the glycan cap.

The glycan cap-specific Abs could neutralize EBOV in several ways: by blocking virion attachment to cells, preventing virus internalization, or inhibiting GP cleavage by host cathepsins, which is required to unmask the NPC1 receptor-binding site. To test whether neutralizing Abs to the glycan cap interfere with virus attachment to host cells, the target Abs can be tested in pre-attachment neutralizing assays where mAbs are pre-incubated with virus or virus-like particles at 4°C before being inoculated onto cell monolayers (1 hour at 4°C). The virus or virus-like particles/antibody mixtures then can be placed onto cell culture monolayers and incubated for 1 hr at 4°C. After non-absorbed virus or virus-like particles are removed, cell monolayers can be overlaid with media containing methylcellulose and incubated for 48 hrs at 37°C. Plaques can be counted using a fluorescence microscope.

The glycan cap-specific antibodies described here bind to sites distant from the putative cathepsin cleavage site (located at residue 190), so they are unlikely to interfere with GP cleavage. To test whether glycan-cap specific mAbs inhibit the proteolytic cleavage of EBOV surface protein, EBOV GP could be incubated with the mAbs for 1 hour at 37°C and then treated with cathepsins B and L for an additional hour. The cleavage of EBOV GP can be verified in western blot by the presence of a 19 kDa cleaved form of GP.
Mechanisms of *Ebolavirus* neutralization by HR2/MPER-specific mAbs

Multiple neutralizing mAbs bind to the GP base, including two of three murine-origin Abs from the ZMapp™ cocktail (c2G4 and c4G7) and the human neutralizing antibody obtained from a phage-display library (KZ52). The epitopes of these mAbs were mapped to a conformationally-sensitive region at the GP1/GP2 interface in the GP base. These GP1/GP2 interface-specific Abs might neutralize EBOV by blocking fusogenic rearrangement and membrane insertion by GP2, where the HR1 sequence in the pre-fusion GP2 (GP2pre, Figure 31) rearrange to form an unbroken α-helix (GP2extended), placing the GP2 internal fusion loop into the target membrane.

In Chapter IV, I describe three neutralizing human Abs (BDBV223, BDBV317 and BDBV340) that bind to a new antigenic region in the HR2/MPER region of GP2. We used a comprehensive alanine-scanning approach and identified critical residues in GP2 for these neutralizing Abs. The critical residues spanned the C-terminal part of HR2 region and the N-terminal part of the MPER which form an α-helix. Therefore, in contrast to GP1/GP2 interface-specific Abs that block the transition from GP2pre to GP2extended conformation, HR2/MPER-specific Abs might block the further rearrangement of GP2 from extended conformation to the six-helix bundle (GP2\(_{6HB}\)), where HR2 packs against trimeric HR2 coiled coil to form the six-helix bundle (Figure 32).

To define the residues in the HR2 α-helix required for binding by HR2/MPER-specific mAbs, crystallographic studies could be performed with cross-reactive mAbs complexed with either recombinant EBOV GP or HR2/MPER peptides. Comparison of crystal structures of neutralizing and protective mAbs (BDBV223 and BDBV317) with the structure of non-protective mAb BDBV340 could further inform the development of an HR2/MPER-based EBOV vaccine effective against multiple filoviruses.
A real-time assay for EBOV GP triggering and lipid mixing has been described (Spence et al., 2016). This system uses the VSV particles bearing EBOV GP, a fluorescent monomeric NeonGreen protein fused to EBOV phosphoprotein, and lipophilic dye DiD (1,1'-Dioctadecyl-3,3',3''-Tetramethylindodicarbocyanine), which is incorporated into the viral membrane. When incorporated into virions, DiD displays fluorescent self-quenching. Lipid mixing between viral and host membranes enables lateral diffusion of DiD dye, which yields a sharp increase in the fluorescent signal. Such real-time systems could be used to determine whether HR2/MPER-specific mAbs inhibit conformational rearrangements of GP2.
that ultimately lead to the fusion of viral and host membranes. The HR2/MPER-specific mAbs could be tested in the presence or absence of GP1/GP2 interface-specific mAbs to investigate the type of interaction between mAbs that target two separate antigenic regions on the GP base (additive, synergistic, or antagonistic).

B cell repertoire diversities of filovirus-specific antibodies

High-throughput sequencing technologies can be used to explore Ab gene signatures in humans (Arnaout et al., 2011; Wu et al., 2010). For some neutralizing epitopes, the Ab repertoire is restricted to a limited set of Ab genes (Scheid et al., 2011). Convergent Ab gene rearrangement signatures of the dengue virus-specific B-cell population occur during the acute phase of dengue virus infection (Parameswaran et al., 2013). It is not known whether the limited number of neutralizing epitopes on EBOV or MARV GPs can serve as a constraint mediating the development of convergent Ab sequences that are specific to memory B-cell populations of filovirus survivors.

To analyze the Ab repertoire of filovirus survivors, total RNA could be extracted from PBMC samples of BDBV survivors and, after cDNA synthesis, PCR amplification could be performed with mixtures of primers designed to amplify antibody gene segments (Smith et al., 2009). The purified PCR products could be submitted for high-throughput sequencing using an Illumina platform. The resulting sequence data could be analyzed using the clonal lineage identification methods to identify filovirus-specific signatures in the B-cell repertoire of survivors. The phylogenetic analysis could be performed using nucleotide sequences obtained from hybridomas expressing filovirus-specific neutralizing Abs to find sequence families for Ab somatic variants. Ab variants identified by high-throughput sequence analysis could be tested in a binding assays with MARV and EBOV GPs.


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Highlights

- Marburg virus survivor-neutralizing antibodies bind to a single antigenic site
- Several of the survivors’ antibodies also bind to Ebola virus glycoprotein
- All antibodies identified bind at the predicted region of the receptor-binding site
- Binding to receptor-binding site is a new mechanism of filovirus inhibition

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In Brief

The characterization of Marburg-specific antibodies in several patients who survived the infection reveals a common binding site in the viral glycoprotein and a mechanism for filovirus inhibition.
Mechanism of Human Antibody-Mediated Neutralization of Marburg Virus


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SUMMARY
The mechanisms by which neutralizing antibodies inhibit Marburg virus (MARV) are not known. We isolated a panel of neutralizing antibodies from a human MARV survivor that bind to MARV glycoprotein (GP) and compete for binding to a single major antigenic site. Remarkably, several of the antibodies also bind to Ebola virus (EBOV) GP. Single-particle EM structures of antibody-GP complexes reveal that all of the neutralizing antibodies bind to MARV GP at or near the predicted region of the receptor-binding site. The presence of the glycan cap or mucin-like domain blocks binding of neutralizing antibodies to EBOV GP, but not to MARV GP. The data suggest that MARV-neutralizing antibodies inhibit virus by binding to infectious virions at the exposed MARV receptor-binding site, revealing a mechanism of filovirus inhibition.

INTRODUCTION
Marburg virus (MARV) and Ebola virus (EBOV), which are members of the family Filoviridae, infect humans and non-human primates, causing a hemorrhagic fever with mortality rates up to 90% (Brauburger et al., 2012). There have been a dozen outbreaks of Marburg virus infection in humans reported to date, including the most recent report from Uganda of a 30-year-old male health worker who died in September 2014 (WHO, 2014a). As of January 7, 2015, there have been in excess of 20,000 confirmed, probable, and suspected cases of Ebola virus disease (EVD) in the current EBOV outbreak in nine affected countries (Guinea, Liberia, Mali, Nigeria, Senegal, Sierra Leone, Spain, the United Kingdom, and the United States of America), with more than 8,000 deaths (WHO, 2014b).

There is no licensed treatment or vaccine for filovirus infection. Recently, several studies showed that filovirus glycoprotein (GP)-specific neutralizing antibodies (nAbs) can reduce mortality following experimental inoculation of animals with a lethal dose of EBOV (Dye et al., 2012; Marzi et al., 2012; Olinger et al., 2012; Qiu et al., 2012, 2014; Pettitt et al., 2013) or MARV (Dye et al., 2012). The primary target of these nAbs, the filovirus surface GP, is a trimer composed of three heavily glycosylated GP1-GP2 heterodimers (Figure S1). The GP1 subunit can be divided further into base, head, glycan cap, and mucin-like domains (Lee et al., 2008). During viral entry, the mucin-like domain and glycan cap mediate binding to multiple host attachment factors present on the cell membrane. After the virus enters the host cell by macropinocytosis (Nanbo et al., 2010; Saeed et al., 2010), the GP is cleaved by host proteases that remove approximately 80% of the mass of the GP1 subunit, including the mucin-like domain and glycan cap (Chandran et al., 2005; Dube et al., 2009). After cleavage of GP in the endosome, the receptor-binding sites on GP become exposed, and the GP1 head then is able to bind to its receptor, Niemann-Pick C1 (NPC1) protein (Carette et al., 2011; Chandran et al., 2005; Côté et al., 2011). Subsequent conformational changes in GP facilitate fusion between viral and endosomal membranes.

The dense clustering of glycans on the glycan cap and mucin-like domain likely shield much of the surface of EBOV GP from humoral immune surveillance, leaving only a few sites on the EBOV GP protein at which nAbs could bind without interference by glycans (Cook and Lee, 2013). Most of our knowledge about humoral response against filovirus infections has come from studies of murine Abs that recognize EBOV GP. From those studies, we learned that mouse nAbs preferentially target peptides exposed in upper, heavily glycosylated domains or lower areas (the GP1 base), where rearrangements occur that drive
fission of viral and host membranes (Saphire, 2013). Abs have not been identified that target protein features of the GP1 head sub-domain, where the receptor-binding site to NPC1 protein is located. Ab KZ52, the only reported human EBOV GP-specific mAbs, was obtained from a phage display library that was constructed from bone marrow RNA obtained from a survivor (Maruyama et al., 1999). KZ52 binds a site at the base of the GP and neutralizes EBOV, most likely by inhibiting the conformational changes required for fusion of viral and endosomal membranes (Lee et al., 2008). Some murine Abs also have been reported to bind to the base region of Ebola virus GP (Dias et al., 2011, Murin et al., 2014). In contrast, very little is known about the mechanisms by which Abs neutralize MARV. Two murine Abs that bound the mucin-like domain of MARV GP reduced MARV budding from infected cells in culture but failed to neutralize virus directly (Kajihara et al., 2012). Polyclonal MARV-specific Abs were shown to protect non-human primates when administrated passively after challenge (Dye et al., 2012). The epitopes recognized by such polyclonal nAbs, and the mechanism of neutralization by which these Abs act, are unknown. In this study, we isolated a large panel of human nAbs from B cells of a human survivor of severe MARV infection and used these Abs to define the molecular basis of MARV neutralization by human Abs. The results show that MARV nAbs recognize the NPC1 receptor-binding domain of MARV GP and, in some cases, also recognize conserved structural features in the equivalent receptor-binding domain on EBOV GP.

RESULTS

Isolation of Monoclonal Antibodies

We tested plasma of a MARV survivor previously infected in Uganda for the 50% neutralization activity against the Uganda strain of MARV and found a serum-neutralizing titer of 1:1,010. To generate human hybridoma cell lines secreting mAbs to MARV, we screened supernatants from EBV-transformed B cell lines derived from the survivor for binding to several recombinant forms of MARV GP or to irradiated cell lysates prepared from MARV-infected cell cultures. We fused transformed cells from B cell lines producing MARV-reactive Abs to the MARV antigens with myeloma cells and generated 51 cloned hybridomas secreting MARV-specific human mAbs. Thirty-nine of these Abs were specific to the MARV GP, while 12 bound to infected-cell lysate, but not to GP; these latter mAbs were shown in secondary screens to bind to MARV internal proteins (NP, VP35, or VP40; data not shown). Analysis of the Ab heavy- and light-chain variable domain sequences revealed that all MARV-specific mAbs were encoded by unique Ab genes.

Neutralization Activity

To evaluate the inhibitory activity of the mAbs, we first performed in vitro neutralization studies using a chimeric vesicular stomatitis virus with MARV GP from Uganda strain on its surface (vesicular stomatitis virus/Marburg glycoprotein recombinant VSV/GP-Uganda). Eighteen of the 39 MARV GP-specific mAbs exhibited neutralization activity against VSV/GP-Uganda (Figures 1A and 1C; Figures S2 and S4). Of those 18 nAbs, 9 displayed strong (IC50 < 10 μg/ml), 8 nAbs displayed moderate (IC50: 10–99 μg/ml), and one displayed weak (IC50: 100–1,000 μg/ml) neutralizing activity against VSV/GP-Uganda. We also tested the neutralization potency of all nAbs that bound to MARV GP in a plaque reduction assay using live MARV-Uganda virus. Of 18 Abs that neutralized VSV/GP-Uganda, 11 Abs exhibited neutralizing activity against MARV-Uganda (Figures 1A and 1C; Figures S3 and S4). These data suggest that VSV/GP, often used to study neutralizing potency of Abs because of its BSL-2 containment level, is more susceptible to Ab-mediated neutralization than live MARV. This difference is likely explained by the significantly lower copy number of MARV GP molecules that incorporate into VSV particles compared with the large number of GP molecules on the surface of filovirus filaments (Beniac et al., 2012; Thomas et al., 1985). Comparison of MARV-neutralizing and non-neutralizing antibodies at concentration up to 1.6 mg/ml revealed dose-dependent activity of those mAbs that neutralized. The neutralization activity of nAbs was not enhanced by the presence of complement (data not shown). As expected, we did not detect neutralizing activity for any of the 12 Abs specific to MARV NP, VP35, or VP40 proteins.

Recognition of Varying Forms of GP

To characterize the binding of isolated Abs to recombinant MARV GPs, we performed binding assays using either a recombinant MARV GP ectodomain containing the mucin-like domain (MARV GP) or a recombinant GP lacking residues 257–425 of the mucin-like domain (MARV GPmuc). Based on OD405 values at the highest Ab concentration tested (Emax) and 50% effective concentration (EC50), we divided the MARV-GP-specific Abs into four major groups, based on binding phenotype (designated binding groups 1, 2, 3A, and 3B; Figures 1B and S5). Binding group 1 mAbs had an Emax to GP <2 (i.e., these mAbs never exhibited a maximal binding level to MARV GP); binding group 2 mAbs had an Emax to GP >2, with EC50 for GP < EC50 for GPmuc (i.e., these mAbs bound to the mucin-like domain or glycan cap); and binding group 3 had an Emax to GP >2, with EC50 for GP = EC50 for GPmuc (i.e., these mAbs bound equally well to full-length and mucin-deleted forms of GP), with the group 3A mAbs having an EC50 for GP < 0.5 μg/ml and the group 3B mAbs having an EC50 for GP > 0.5 μg/ml (suggesting that, as a class, the group 3B mAbs possess a lower steady-state Kd of binding to GP than did group 3A mAbs).

Abs that lacked neutralization activity against VSV/GP-Uganda or MARV-Uganda fell principally into binding groups 1, 2, and 3A. Interestingly, all VSV/GP-Uganda nAbs displayed a unique binding pattern and segregated into binding group 3B (Figure 1C). It was interesting that while both mAbs from groups 3A and 3B bound equally well to the full-length MARV GP and to the GPmuc, EC50 values for nAbs from binding group 3B were higher than those for non-neutralizing Abs from group 3A.

Competition-Binding Studies

To determine whether mAbs from distinct binding groups targeted different antigenic regions on the MARV GP surface, we performed a competition-binding assay using a real-time biosensor. We tested 18 MARV nAbs from binding group 3B, 4 Abs from binding group 3A, and 1 Ab from binding group 2 in a tandem blocking assay in which biotinylated GPmuc was
attached to a streptavidin biosensor. Abs from group 1 and the two non-neutralizing Abs from binding group 3B did not bind to biotinylated GPΔmuc in the competition assay and were excluded from the analysis. While non-neutralizing Abs from binding groups 2 and 3A did not prevent binding of the binding group 3B mAbs to GPΔmuc, all nAbs blocked binding of each of the other nAbs to the antigen and segregated into a single competition-binding group (Figure 1D). These data suggested...
that all of the nAbs target a single major antigenic region on the MARV GP surface.

**Electron Microscopy Studies of Antigen-Antibody Complexes**

To determine the location of the antigenic region targeted by MARV nAbs, we performed negative stain single-particle electron microscopy (EM) studies using complexes of GP$_{\Delta}$Muc with Fab fragments of seven nAbs from Binding Group 3B. The EM reconstructions clearly showed that Fab fragments for all seven nAbs bind at the top of the GP in or near the NPC1 protein receptor-binding site (Figures 2A and 2B). The binding pattern of these Abs could be subdivided further into two major groups based on their relative angle of approach to the GP head domain. MAbs MR72, MR78, MR201, and MR82 bound toward the top and side of GP1 at a shallow angle relative to the central 3-fold axis, while mAbs MR191, MR111, and MR198 bound at a steeper angle toward the top of GP1 (Figures 2C and 2D). When we compared IC$_{50}$ values for nAbs that bound in the two binding poses, we did not detect a significant difference in neutralization potency based on the angle of approach (Figure 1C).

**Antibody Neutralization Escape Mutant Viruses**

As an additional strategy to determine residues on MARV GP involved in binding to nAbs, we generated VSV/GP-Uganda variant viruses that escaped neutralization, and then we determined the sequence of the GP of those mAb escape viruses. Vero E6 cells were inoculated with VSV/GP-Uganda in the presence of MR72 or MR78 nAbs. Two escape mutant viruses were isolated: virus variant VSV/GP-72.5 contained three missense mutations in the MARV GP gene (N129S in the putative NPC1 receptor-binding site, S220P in the glycan cap and P455L in the...
mucin-like domain), and virus variant VSV/GP-78.1 possessed missense mutation C226Y in the glycan cap (Figure 3A). Consistent with the EM data, six out of seven nAbs tested displayed a higher level of neutralization activity against the wild-type VSV/GP-Uganda than to the VSV/GP-72.5 or VSV/GP-78.1 escape mutant viruses, suggesting these nAbs recognize MARV GP in a similar fashion (Figure 3B). MAb MR198 exhibited equal neutralization potency against wild-type VSV/GP-Uganda or the two escape mutant viruses (Figure 3B). As all nAbs segregated into one competition group (Figure 1D), bound the MARV GP at the NPC1 receptor-binding site (Figures 2A–2D), and displayed a similar profile of neutralization of escape mutant viruses (Figure 3B), we propose that blocking of MARV GP binding to NPC1 is the principal mechanism of MARV neutralization by these naturally occurring human Abs. This model is supported by the data in the accompanying paper by Hashiguchi et al. (2015; this issue of Cell) showing that MR78 inhibits binding of NPC1 domain C to MARV GP.

Cross-Reactive Binding of MARV Antibodies with EBOV GP

It is surprising that human MARV nAbs recognize the putative NPC1 protein receptor-binding site on GP, since previous studies suggested that the NPC1 protein receptor-binding site on EBOV GP may be obscured from Ab binding by the presence of the highly glycosylated glycan cap and mucin-like domain (Lee et al., 2008). To determine whether the MARV nAbs we isolated also could bind in a cross-reactive manner to the EBOV GP receptor-binding site, we performed ELISA using three recombinant forms of MARV and EBOV GPs: full-length GP ectodomain containing the glycan cap and mucin-like domain (designated MARV or EBOV GP), ectodomains lacking residues 257–425 (MARV) or 314–462 (EBOV) of the mucin-like domain (designated MARV or EBOV GP\_muc), and cleaved GP ectodomains enzymatically treated to remove the mucin-like domain and glycan cap (designated MARV or EBOV GPcl). Three of the MARV nAbs, designated MR78, MR111, and MR191, recognized the EBOV GPcl that lacked the glycan cap and mucin-like domain (Figure 4A). Remarkably, the MARV nAb MR72 bound all three forms of both EBOV and MARV GPs with similar EC50 and Emax values, indicating that its epitope, and the EBOV receptor-binding site, which it likely overlaps, might be partially accessible for Ab binding even in the full-length form (Figure 4A). We tested the breadth of neutralization of MARV nAbs for filoviruses using a panel of different MARV and EBOV isolates. While multiple MARV Abs displayed neutralizing activity toward different MARV strains, MARV nAbs did not exhibit detectable neutralization activity against EBOV or VSV/EBOV (Figure 4B). Structural analysis of MARV and EBOV GP in the accompanying paper by Hashiguchi et al. (2015) reveals that the glycan cap and mucin-like domain likely obscure the receptor-binding domain in EBOV, but not in MARV.

In Vivo Testing

We tested the in vivo protective activity of the mAbs in a murine model using mouse-adapted MARV strain Ci67 (Warfield et al., 2007, 2009). Inoculation of mice with MARV Ci67 causes clinical disease and, in a proportion of animals, causes lethal disease, although typically less than 100% lethality in mice (Warren et al., 2014). We selected four of the mAbs among those with the lowest in vitro neutralization IC50 values: MR72, MR82, MR213, and MR232. The IC50 values in neutralization assays with MARV Uganda or mouse-adapted MARV strain Ci67 were comparable (within 2-fold). Seven-week-old BALB/c mice were
by >. Neutralization assays were performed in triplicate.

### Table A: Binding (µg/mL)

<table>
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<th>mAb</th>
<th>MARV</th>
<th>EBOV</th>
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<tr>
<td></td>
<td>GP</td>
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### Table B: Neutralization (µg/mL)

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<th>VSV/GP-Uganda</th>
<th>MARV-Musoke</th>
<th>MARV-Uganda</th>
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<th>MARV-Ravn</th>
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Figure 4. Breadth of Binding or Neutralization of Human MARV-Specific mAbs for Diverse Filoviruses

(A) A heatmap showing the binding in ELISA of neutralizing mAbs from binding group 3B to the MARV and EBOV GPs. EC_{50} value for each antigen-mAb combination is shown, with dark red shading indicating lower EC_{50} values or orange or yellow shading indicating intermediate or higher EC_{50} values. EC_{50} values greater than 1,000 µg/ml are indicated by >.

(B) A heatmap showing the neutralization breadth of mAbs from binding group 3B. The IC_{50} value for each virus-mAb combination is shown, with dark red shading indicating increased potency and orange or yellow shading indicating intermediate or low potency. IC_{50} values greater than 1,000 µg/ml are indicated by >. Neutralization assays were performed in triplicate.

Injected with 100 µg of antibody by the IP route and challenged with 1,000 plaque-forming unit (PFU) of Ci67. Twenty-four hours later, antibody treatment was repeated. By day 6, all five control (untreated) mice developed progressive loss of weight and symptoms of the disease, including dyspnea, recumbency, and unresponsiveness, and on days 8 and 9, two animals were found dead and one animal was found moribund and euthanized. The remaining two animals demonstrated recovery by day 11. In contrast, all animals treated with any antibody survived and did not display the elevation of the disease score, with the exception of two animals treated with MR72, which showed a transient marginal loss of weight and increase of the disease score on days 6–9, which did not exceed 1 (Figure 5). The observed level of protection was remarkable given the relatively modest intravital-neutralizing potency of the antibodies.

**DISCUSSION**

There is an obvious urgent need for prophylactic and therapeutic interventions for filovirus infections given the recurrence of MARV outbreaks, including that in Uganda in October 2014 and a massive outbreak of EBOV infections in West Africa in 2014. There is very little information about the structural determinants of neutralization on which to base the rational selection of antibodies, and for MARV there have been no reported human nAbs.

This study reveals that naturally occurring human MARV nAbs isolated from the B cells of a recovered donor principally target the MARV NPC1 protein receptor-binding site, suggesting that a major mechanism of MARV neutralization could be inhibition of binding to receptor. Remarkably, some of the isolated anti-bodies also bound to the EBOV GP. This mechanism of MARV neutralization was unexpected, because previous studies with EBOV showed that the putative receptor-binding domain on GP is obscured on the surface of virions by the presence of the glycan cap and mucin-like domain, only becoming exposed following cleavage by cathepsin in the endosome. These studies suggest that the configuration of the MARV GP differs significantly from that of EBOV GP because the receptor-binding domain must be accessible for immune recognition on MARV GP. Indeed, determination of the structure of the MARV GP and structural analysis of the interaction of mAb MR78 with MARV and EBOV GP molecules shows this to be the case (see Hashiguchi et al., 2015).

The information obtained from these studies can be used to inform development of new therapeutics and structure-based vaccine designs against filoviruses. Furthermore, as these nAbs are fully human and exhibit inhibitory activity, they might be useful as a component of a prophylactic or therapeutic approach for filovirus infection and disease. The challenge studies using a murine model here show clear evidence of in vivo activity and suggest additional preclinical studies in other species, such as guinea pigs and macaques, are warranted. Their ability to bind a broad range of MARV isolates indicates they may offer detection of or efficacy against new viral strains yet to emerge. Although some of these mAbs bind to certain forms of EBOV GP, these antibodies are not likely to be effective against natural Ebola infection because the EBOV receptor-binding site is obscured on the viral surface. However, such mAbs might neutralize EBOV if they could be delivered to the endosome, where the EBOV receptor-binding site is exposed following GP cleavage.
ELISA, viruses were gamma-irradiated with the dose of 5 \( \times 10^6 \) rad. The recombinant Ebola Zaire strain Mayinga (EBOV) expressing eGFP was generated in our laboratory by reverse genetics (Lubaki et al., 2013; Towner et al., 2009) from plasmids provided by the Special Pathogens Branch at CDC and passed five times in Vero E6 cells. The plasmid pVSV/GP-Uganda. To recover the recombinant virus, 1 \( \mu g \) of RNA was isolated and reverse transcribed. MARV GP open reading frame (ORF) was PCR amplified from cDNA using forward primer 5'-CATGTACGAAGGGCATGAGGACTA-3' and reverse primer 5'-TCTAGCAGCTCAGGTTCAACATGAGGACTA-3'.

Figure 5. Survival and Clinical Overview of Mice Treated with MARV mAbs
(A–C) Groups of mice at five animals per group were injected with individual mAbs by the intraperitoneal route twice: 1 hr prior and 24 hr after MARV challenge at 100 \( \mu g \) per treatment. Untreated animals served as controls. (A) Kaplan-Meier survival curves. (B) Body weight. (C) Illness score.

**EXPERIMENTAL PROCEDURES**

**Donor**
The donor was an otherwise healthy adult woman who contracted Marburg virus (MARV) infection in 2008 following exposure to fruit bats in the Python Cave in Queen Elizabeth National Park, Uganda. The donor’s clinical course was documented previously (CDC, 2009). Peripheral blood from the donor was obtained in 2012, four years after the illness, following informed consent. The study was approved by the Vanderbilt University Institutional Review Board.

**Viruses**
MARV strain 200702854 Uganda (MARV-Uganda) was isolated originally from a subject designated “patient A” during the outbreak in Uganda in 2007 (CDC, 2009; Towner et al., 2009) and underwent four passages in Vero E6 cells. MARV strain Musoke (MARV-Musoke) was isolated during the outbreak in Kenya in 1987 (Smith et al., 1982) and passaged five times in Vero E6 cells. MARV strain 200501379 Angola (MARV-Angola) was isolated during the outbreak in Angola in 2005 (Towner et al., 2006) and passaged three times in Vero E6 cells. MARV Ravn virus (Ravn) was isolated from a patient in 1987 in Kenya (Johnson et al., 1996) and passaged four times in Vero E6 cells. All strains of MARV were obtained originally from the Special Pathogens Branch, U.S. Centers for Disease Control (CDC), and deposited at the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) housed at UTMB. The recombinant Ebola Zaire strain Mayinga (EBOV) expressing eGFP was generated in our laboratory by reverse genetics (Lubaki et al., 2013; Towner et al., 2009) from plasmids provided by the Special Pathogens Branch at CDC and passaged three times in Vero E6 cells. For analysis of antibody binding by ELISA, viruses were gamma-irradiated with the dose of 5 \( \times 10^6 \) rad.

The donor was an otherwise healthy adult woman who contracted Marburg virus (MARV) infection in 2008 following exposure to fruit bats in the Python Cave in Queen Elizabeth National Park, Uganda. The donor’s clinical course was documented previously (CDC, 2009). Peripheral blood from the donor was obtained in 2012, four years after the illness, following informed consent. The study was approved by the Vanderbilt University Institutional Review Board.

**Generation of a Chimeric Strain of VSV in which VSV G Protein Was Replaced with the GP Protein of MARV Strain Uganda**
The plasmid pVSV-XN2 carrying cDNA of the full-length VSV anti-genome sequence and the support plasmids pBS-N, pBS-L, and pBS-P encoding the internal VSV proteins under control of the T7 promoter were kindly provided by Dr. Yoshihiro Kawaoka (University of Wisconsin). For generation of the VSV/GP-Uganda construct, Vero E6 cell monolayers were inoculated with MARV strain 200702854, and total cellular RNA was isolated and reverse transcribed. MARV GP open reading frame (ORF) was PCR amplified from cDNA using forward primer 5'-5CATGTACGAAGGGCATGAGGACTA-3' and reverse primer 5'-TCTAGCAGCTCAGGTTCAACATGAGGACTA-3'. The Mouse-adapted Ci67 strain of Marburg virus (Warfield et al., 2007) was provided by Dr. Sina Bavari (U.S. Army Medical Research Institute of Infectious Diseases) and amplified by a single passage in Vero-E6 cells.

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Generation of Human Hybridomas Secreting Monoclonal Antibodies

Peripheral blood mononuclear cells (PBMCs) from the donor were isolated with Ficoll-Histopaque by density gradient centrifugation. The cells were cryopreserved immediately and stored in the vapor phase of liquid nitrogen until use. Previously cryopreserved samples were thawed, and ten million PBMCs were plated into 384-well plates (Nunc #164688) using 17 ml of cell culture medium (ClonaCell-HY Medium A, StemCell Technologies, #03801), 8 μg/ml of the TLR agonist CpG (phosphorothioate-modified oligodeoxynucleotide ZOE2ZOEZOE2ZOEZOEZOEZ2Z, invitrogen), 3 μg/ml of the Chk2 inhibitor (Sigma #CST242), 1 μg/ml of cyclosporine A (Sigma #C11932), and 4.5 ml of clarified supernate from cultures of 895.8 cells (ATCC VR-1492) containing Epstein-Barr virus (EBV). After 7 days, cells from each 384-well culture plate were expanded into four 96-well culture plates (Falcon #330727) using cell culture medium containing 8 μg/ml CpG, 3 μg/ml Chk2, and ten million irradiated heterologous human PBMCs (Nashville Red Cross) and incubated for an additional 4 days. Plates were screened for MARV antigen-specific antibody-secreting cell lines using ELISAs. Cells from wells with supernates reacting in a MARV antigen ELISA were fused with HMMA2.5 myeloma cells using an established electroporation technique (Yu et al., 2008). After fusion, hybridomas were resuspended in medium containing 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine (HAT Media Supplement, Sigma #H0262), and 7 μg/ml ouabain (Sigma #O3125) and incubated for 18 days before screening hybridomas for antibody production by ELISA.

Human mAb and Fab Production and Purification

After fusion with HMMA2.5 myeloma cells, hybridomas producing MARV-specific antibodies were cloned biologically by two rounds of limiting dilution and by single-cell fluorescence-activated cell sorting. After cloning, hybridomas were expanded in post-fusion medium (ClonaCell-HY Medium E, STEMCELL Technologies #03805) until 50% confluent in 75-cm² flasks (Corning #430641). Cell monolayers were detached using 3-mm glass beads, and cell suspensions were centrifuged at 1,000 g for 10 min at 4°C. Supernates were discarded; cell pellets were resuspended in 10× excess of borate buffer saline (10 mM Na2B4O7 and 150 mM NaCl [pH 9.0]) and centrifuged at 16,000 × g for 10 min at 4°C. Supernates were discarded; cell pellets were resuspended in 10× excess of borate buffer saline (10 mM Na2B4O7 and 150 mM NaCl [pH 9.0]) and centrifuged at 16,000 × g for 10 min at 4°C. Supernates were discarded; cell pellets were resuspended in cold 1% Triton X-100 (Fisher Scientific) in borate buffer saline, vortexed, and gamma-irradiated on dry ice at 5 × 10⁹ rad. The lysates were sonicated with a 600 W Tekmar Sonic Disruptor TM600 (Tekmar) using a cuphorn sonicator at maximum power setting and 50% duty cycle for 10 min and centrifuged at 16,000 × g, and the supernates were aliquoted.

Screening ELISA

ELISA plates were coated with lysates of MARV-infected cells (diluted 1:1,000 in Dulbecco’s PBS [DPBS]) or recombinant MARV GP or MARV GPΔmuc proteins (20 μg in 10 ml DPBS per plate) and incubated at 4°C overnight. Plates were blocked with 100 μl of blocking solution/well for 1 hr. Blocking solution consisted of 10 g powdered milk, 10 ml of goat serum, 100 ml of 10× DPBS, and 0.5 ml of Tween-20 mixed to a 1:1 final volume with distilled water. The presence of antibodies bound to the GP was determined using goat anti-human immunoglobulin G (IgG) horseradish peroxidase-conjugated secondary antibodies (Southern Biotech #2040-05, 1:4,000 dilution) and 1-Step Ultra TMB-ELISA substrate (Thermo Scientific #34029), with optical density read at 450 nm after stopping the reaction with 1M HCl.

Half-Maximal Effective Concentration Binding Analysis

MARV or EBOV GPs, MARV or EBOV GPΔmuc, or Ravn or EBOV cathepsin-cleaved GPs were designed and expressed similarly. Large-scale production of recombinant proteins for Ravn strain cleaved GP, EBOV Mayinga strain GP, EBOV Mayinga strain GPΔmuc, and EBOV Mayinga strain cleaved GP were designed and expressed similarly. Recombinant proteins for Ravn strain cleaved GP, EBOV Mayinga strain GP, EBOV Mayinga strain GPΔmuc, and EBOV Mayinga strain cleaved GP were expressed and purified by chromatography using a two-column system in which the first column contained protein G resin (GE Healthcare Life Sciences #2504501 and #11003494, respectively) were used to purify antibodies from filtered supernates. Fab fragments were generated by papain digestion (Pierce Fab Preparation Kit, Thermo Scientific #49985) and purified by chromatography using a two-column system in which the first column contained protein G resin (GE Healthcare Life Sciences #2504585) and the second column contained either anti-kappa or anti-lambda antibody light chain resins (GE Healthcare Life Sciences #17545811 and #17548211, respectively).

Expression and Purification of MARV and EBOV GPs

Angola strain MARV GP ectodomains, containing the mucin-like domain (MARV GP) or lacking residues 257–425 of the mucin-like domain (MARV GPΔmuc), were used to screen supernates of transformed B cells and human hybridomas separately. Recombinant proteins for Ravn strain cleaved GP, EBOV Mayinga strain GP, EBOV Mayinga strain GPΔmuc, and EBOV Mayinga strain cleaved GP were designed and expressed similarly. Recombinant proteins for Ravn strain cleaved GP, EBOV Mayinga strain GP, EBOV Mayinga strain GPΔmuc, and EBOV Mayinga strain cleaved GP were expressed and purified by chromatography using a two-column system in which the first column contained protein G resin (GE Healthcare Life Sciences #29048581) and the second column contained either anti-kappa or anti-lambda antibody light chain resins (GE Healthcare Life Sciences #17545811 and #17548211, respectively).

Lysates of MARV-Infected Cells

Lysates were prepared as previously described (Ksiazek et al., 1999). Briefly, Vero E6 cell monolayers in 850 cm² roller bottles were inoculated with approximately 10⁶ PFU MARV or EBOV and incubated at 37°C until partial destruction of monolayer occurred (approximately 9–10 days). Cell monolayers were detached using 3-mm glass beads, and cell suspensions were centrifuged at 16,000 × g for 10 min at 4°C. Supernates were discarded; cell pellets were resuspended in 10× excess of borate buffer saline (10 mM Na2B4O7 and 150 mM NaCl [pH 9.0]) and centrifuged at 16,000 × g for 10 min at 4°C. Supernates were discarded; cell pellets were resuspended in cold 1% Triton X-100 (Fisher Scientific) in borate buffer saline, vortexed, and gamma-irradiated on dry ice at 5 × 10⁹ rad. The lysates were sonicated with a 600 W Tekmar Sonic Disruptor TM600 (Tekmar) using a cuphorn sonicator at maximum power setting and 50% duty cycle for 10 min and centrifuged at 16,000 × g, and the supernates were aliquoted.

MARV and EBOV Neutralization Experiments

Dilutions of mAbs in triplicate were mixed with 150 PFU of MARV or EBOV expressing eGFP in MEM containing 10% fetal bovine serum (FBS) (HyClone) and 50 μg/ml gentamicin (Cellgro #30-005-CR) with or without 5% guinea pig complement (MP Biomedicals #642386) in a total volume of 0.1 ml and incubated for 1 hr at 37°C for virus neutralization. Following neutralization, virus-antibody mixtures were placed on monolayers of Vero E6 cells in 24-well plates, incubated for 1 hr at 37°C for virus adsorption, and overlayed with MEM containing 2% FBS and 0.8% methylcellulose (Sigma-Aldrich #M0512). After incubation for 5 days, medium was removed, cells were fixed with 10% formalin (Fisher Scientific #245-884), and plates were sealed in plastic bags and incubated for 24 hr at room temperature. Sealed plates were taken out of the BSL-4 laboratory according to approved SOPs, and monolayers were washed three times with PBS. Viral plaques were immunostained with the serum of rabbits that had been hyperimmunized with MARV, or with a mAb against EBOV, clone 15H10 (BEI Resources #NR-12184). Alternatively, following virus adsorption, monolayers were covered with MEM containing 10% FBS and 1.6% tragaracn (Sigma-Aldrich #G1128). After incubation for 14 days, medium was removed, cells were fixed with 10% formalin, and plates were sealed in plastic bags, incubated for 24 hr at room temperature, and taken out of the BSL-4 laboratory as above. Fixed monolayers were stained with 10% formalin containing 0.25% crystal violet (Fisher Scientific #C581-100), and plates were counted.

VSV-MARV and VSV-EBOV Neutralization Tests

Neutralization assays were performed in triplicate, as described above for MARV and EBOV. Following neutralization, virus-antibody mixtures were placed on monolayers of Vero E6 cells in duplicate, incubated for 1 hr at 37°C for virus adsorption, and overlayed with MEM containing 2% FBS containing 0.9% methylcellulose. After incubation for 3 days, medium was...
removed, monolayers were fixed and stained with 10% formalin containing 0.25% crystal violet, and plaques were counted.

**Generation and Sequencing of VSV/GP-Uganda Escape Mutants**

Vero E6 cell monolayers with 2-fold dilutions of mAbs (12.5–200 μg/ml) added to the medium were inoculated with 200 PFU of recombinant VSV/GP-Uganda and incubated at 37°C for 2–4 days. To determine which samples contained live virus, supernatants were collected, virus was titrated in Vero E6 cell monolayers under methylcellulose overlay, monolayers were incubated at 37°C for 3–4 days, and plaques were counted. Supernatants with the highest concentrations of mAbs, which were found to contain live virus by plaque titration, were incubated in presence of serially diluted mAbs, followed by titration of virus as above. The procedure was performed a total of three times. Escape mutant viruses harvested after the third passage were cloned biologically by plaque purification. For biological cloning, Vero E6 cell monolayers in 24-well plates were inoculated with dilutions of the escape mutant viruses in the presence of the corresponding mAbs (200 μg/ml of MR72 or 100 μg/ml of MR78) and covered with 0.7% low melting temperature SeaPlaque agarose (Lonza #50100). Monolayers were incubated at 37°C for 6 days; plaques were visualized with 0.01% neutral red aqueous solution (Electron Microscopy Sciences), picked, resuspended in medium, and transferred to Vero E6 cell monolayers in 24-well plates in the presence of the corresponding mAbs (200 μg/ml of MR72 or 100 μg/ml of MR78) for virus propagation. In 2–5 days, based on the extent of CPE observed, virus was harvested, and cells were dissolved in Trizol reagent (Life Technologies 15596018). Total cellular RNA was extracted, reverse transcribed, and amplified by PCR with the primers described above for generation of a chimeric strain of VSV. Two overlapping fragments covering MARV GP ORF were PCR amplified from cDNA using forward primer 5'−CATGTACGACGCGTCAACATGAGGACTA−3' and reverse primer 5'−ACT AAGCCCTCCTGTCGCAGGT−3' or forward primer 5'−ACCAACATGTAGCCAGG CAA−3' and reverse primer 5'−TCTAGGACGCTCAGCAGCTACATCCATAATTTAG TAAAGATACCAGCAA−3', and the nucleotide sequences of the GP ORFs were determined using standard procedures.

**Analysis of Growth Kinetics of VSV/GP-Uganda Escape Mutant Viruses**

Vero E6 cell monolayers in 24-well plates were inoculated in triplicate with VSV/GP-Uganda escape mutants or non-mutated virus at an MOI of 0.00025 PFU/cell in the presence of varying concentrations of the corresponding mAbs. Aliquots of medium were collected every 12 hr and frozen for titration at a later time. Titration of virus in aliquots was performed as above, without adding antibodies to the culture medium.

**Biolayer Interferometry Competition Binding Assay**

Biotinylated GP or GPΔmuc (E2-link Micro NHS-PEG2-Biotinylation Kit, Thermo Scientific #21955) (1 μg/ml) was immobilized onto streptavidin-coated biosensor tips (Fortebio #18-5019) for 2 min. After measuring the baseline signal in kinetics buffer (KB: 1× PBS, 0.01% BSA, and 0.002% Tween 20) for 2 min, biosensor tips were immersed into the wells containing primary antibody at a concentration of 100 μg/ml for 10 min. Biosensors then were immersed into wells containing competing mAbs at a concentration of 100 μg/ml for 5 min. The percent binding of the competing mAb in the presence of the first mAb was determined by comparing the maximal signal of competing mAb applied after the first mAb complex to the maximal signal of competing mAb alone. mAbs were judged to compete for binding to the same site if maximum binding of the competing mAb was >30% of its un-competed binding. MAbs were considered non-competing if maximum binding of the competing mAb was >70% of its un-competed binding. A level of 30%–70% of its un-competed binding was considered intermediate competition.

**Sequence Analysis of Antibody Variable Region Genes**

Total cellular RNA was extracted from clonal hybridomas that produced MARV antibodies, and RT-PCR reaction was performed using mixtures of primers designed to amplify all heavy-chain or light-chain antibody variable regions. The generated PCR products were purified and cloned into the pCRII 1.2 plasmid vector (Thermo Scientific, #K1231) for sequence analysis. The nucleotide sequences of plasmid DNAs were determined using an ABI3700 automated DNA sequencer. Heavy-chain or light-chain antibody variable region sequences were analyzed using the IMGT/V-Quest program (Brochot et al., 2008; Giudicelli et al., 2011). The analysis involved the identification of germline genes that were used for antibody production, location of complementary determining regions (CDRs), and framework regions (FRs), as well as the number and location of somatic mutations that occurred during affinity maturation.

**Statistical Analysis**

EC50 values for neutralization were determined by finding the concentration of mAb at which a 50% reduction in plaque counts occurred after incubation of virus with neutralizing antibody. A logistic curve was fit to the data using the count as the outcome and the log-concentration as the predictor variable. The results of the model then were transformed back to the concentration scale. Results are presented as the concentration at the dilution that achieves a 50% reduction from challenge control with accompanying 95% confidence intervals. Each antibody was treated as a distinct analysis in a Bayesian non-linear regression model.

**Sample Preparation for EM Studies**

A RV strain MARV GP mucin-deleted construct (GPΔmuc) was produced by stable cell line expression in Drosophila S2 cells, as described above. Human Fab proteins for MARV-specific antibodies were generated as described above. Fabes were added in molar excess to GPΔmuc and allowed to incubate overnight at 4°C. Complexes then were purified by Superdex 200 size-exclusion chromatography in TBS.

**Electron Microscopy and Sample Preparation**

A 4 μl aliquot of each complex that had been diluted to a concentration of ~0.03 μg/ml with TBS buffer was placed for 15 s onto carbon-coated 400 Cu mesh grids that had been plasma cleaned for 20 s (Gatan), blotted off the edge of the grid, and then immediately stained for 30 s with 4 μl of 2% uranyl formate. The stain was blotted off on the edge of the grid, and the grid was allowed to dry. Data were automatically collected with Legionin (Carragher et al., 2000; Potter et al., 1999; Suloway et al., 2005) using a FEI Tecnai F20 electron microscope operating at 120 keV with an electron dose of 30 e⁻/Å² and a magnification of 52,000× that resulted in a pixel size of 2.6 Å. At the specimen plane when collected with Tietz CMOS 4k × 4k CCD camera. Particle orientations appeared to be generally isotropic, and images were acquired at a constant defocus value of −1.0 μm at 0° stage tilt.

**Image Processing of Protein Complexes**

Particles were picked automatically using DoG Picker (34) and placed into a particle stack using the Appion software (Lander et al., 2009). Reference-free 2D class averages were generated with the Xmipp clustering 2D alignment software (van Heel et al., 1996) and sorted into an initial 300 classes. Non-GP particles were removed, and the stack was further subclassified into classes with ~100 particles per class in order to generate the final particle stack used for the reconstruction. Various numbers of class averages were chosen to create initial models using EMAN2 common lines software (Tang et al., 2007). A model that best matched its projected classes was then used for refinement against the raw particle stack, imposing C3 symmetry, and the reconstruction was generated with ten rounds of refinement and increasingly smaller angular sampling rates with EMAN2. All model fitting and manipulation was completed using UCSF Chimera (Pettersen et al., 2004).

**In Vivo Testing**

The animal protocol for testing of mAbs in mice was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston. Seven-week-old BALB/c mice (Harlan) were placed in the ABSL-4 facility of the Galveston National Laboratory. Groups of mice at five animals per group were injected with individual mAbs by the intraperitoneal route twice: 1 hr prior and 24 hr after MARV challenge, using 100 μg per treatment. Untreated animals served as controls. For the challenge, mice were injected with 1,000 PFU of the mouse-adapted MARV strain Ci67 by the intraperitoneal route. Animals were weighed and monitored daily over the 3-week period after challenge. Once animals were symptomatic, they
were examined twice per day. The disease was scored using the following parameters: dyspnea (possible scores 0–5), recumbency (0–9), unresponsiveness (0–5), and bleeding/hemorrhage (0–5); the individual scores for each animal were summarized.

ACCESSION NUMBERS

EM reconstructions have been deposited in the Electron Microscopy Data Bank under the accession codes EMD-6232 through 6238.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.01.031.

AUTHOR CONTRIBUTIONS

A.I.F., P.A.I., and C.D.M. planned, performed, and analyzed experiments and wrote the paper. T.G., X.S., C.K., M.L.F., T.H., Z.A.B., and G.S. performed and analyzed experiments. J.C.S. performed statistical analysis. T.G.K. and A.B.W. planned and analyzed experiments. E.O.S., A.B., and J.E.C. planned and analyzed experiments and wrote the paper.

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effectively neutralized by antibody produced in natural human infection. J. Virol. 73, 6024–6030.


Structural Basis for Marburg Virus Neutralization by a Cross-Reactive Human Antibody

Highlights

- Structure of the Marburg virus GP bound by MR78, a cross-reactive human antibody

- The epitope is conserved among filoviruses and is the likely receptor-binding site

- The antibody–GP interaction mimics that made by the Ebola virus glycan cap

- Mucin domain structure may cause mAbs to react to Ebola and Marburg differently

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In Brief

The structures of Marburg virus glycoprotein in complex with a cross-reactive human antibody, as well as of the Ebola virus glycoprotein bound to the same antibody, reveal that there is a conserved epitope among filoviruses that overlaps with the putative receptor-binding site. These studies provide a map by which therapy with cross-reactive antibodies and inhibitors of entry could be developed.

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Structural Basis for Marburg Virus Neutralization by a Cross-Reactive Human Antibody

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SUMMARY

The filoviruses, including Marburg and Ebola, express a single glycoprotein on their surface, termed GP, which is responsible for attachment and entry of target cells. Filovirus GPs differ by up to 70% in protein sequence, and no antibodies are yet described that cross-react among them. Here, we present the 3.6 Å crystal structure of Marburg virus GP in complex with a cross-reactive antibody from a human survivor, and a lower resolution structure of the antibody bound to Ebola virus GP. The antibody, MR78, recognizes a GP1 epitope conserved across the filovirus family, which likely represents the binding site of their NPC1 receptor. Indeed, MR78 blocks binding of the essential NPC1 domain C. These structures and additional small-angle X-ray scattering of mucin-containing MARV and EBOV GPs suggest why such antibodies were not previously elicited in studies of Ebola virus, and provide critical templates for development of immunotherapeutics and inhibitors of entry.

INTRODUCTION

The filovirus family includes Marburg virus and five ebolaviruses (Ebola, Sudan, Reston, Bundibugyo, and Tai Forest viruses), most of which cause highly lethal hemorrhagic fever and multiple outbreaks among humans. Among the filoviruses, Marburg virus was the first to be identified when it sickened laboratory workers in Europe in 1967 (Malherbe and Strickland-Cholmley, 1968; Siegert et al., 1968). Marburg virus has since re-emerged multiple times, with modern strains conferring greater lethality (~90%) (Geisbert et al., 2007; Towner et al., 2009). Sudan virus has caused at least six outbreaks between 1976 and 2013 (Albaradino et al., 2013; Bowen et al., 1977; Sanchez and Rollin, 2005; Shoemaker et al., 2012), Bundibugyo virus emerged in 2007 (Towner et al., 2008; Wamala et al., 2010) and again in 2012 (Albaradino et al., 2013), and Reston virus was found to infect ranches of swine being raised for human consumption in Asia in 2009 and 2011 (Barrette et al., 2009; Pan et al., 2014; Sayama et al., 2012). Ebola virus is typically found in Central Africa, but re-emerged in Western Africa in 2014 to cause an outbreak unprecedented in magnitude and geographic spread (WHO Ebola Response Team, 2014). During this outbreak, an experimental Ebola virus-specific monoclonal antibody (mAb) cocktail (Qiu et al., 2014) was used compassionately in several patients. No such treatment yet exists that could be used against Marburg virus or the other four ebolaviruses.

Filoviruses express a single protein on their envelope surface, a glycoprotein termed GP, which is responsible for attachment to, and entry of, host cells (Sanchez et al., 1996). GP forms a trimer on the viral surface. In the trimer, each monomer is comprised of GP1 and GP2 subunits that are anchored together by a GP1-GP2 disulfide bond (Volchkov et al., 1998). GP1 contains a receptor-binding core topped by a glycan cap and a heavily glycosylated mucin-like domain (Lee et al., 2008), while GP2 contains two heptad repeats and a transmembrane domain. Filoviruses initially enter cells via macropinocytosis (Alesandrowicz et al., 2011; Nanbo et al., 2010; Saeed et al., 2010; Mulherkar et al., 2011). Once in the endosome, the viral surface GP is cleaved by host cathepsins. Cleavage removes the mucin-like domains and glycan cap (Chandran et al., 2005; Schomberg et al., 2006; Hood et al., 2010; Marzi et al., 2012a; Brecher et al., 2012) and renders GP competent to bind the Niemann Pick C1 (NPC1) receptor (Carete et al., 2011; Coté et al., 2011). Interestingly, Ebola virus entry requires cleavage by cathepsin B (Chandran et al., 2005; Martinez et al., 2010; Schomberg et al., 2006), while Marburg virus entry is independent of cathepsin B (Gnirss et al., 2012; Misasi et al., 2012). The reasons underlying these differences are unknown. After enzymatic cleavage and receptor binding, the GP2 subunit unwinds from its GP1 clamp and rearranges irreversibly into a six-helix bundle (Malashkevich et al., 1999; Weissenhorn et al., 1998a, b) to drive fusion of virus and host membranes.
Antibody therapies recently have demonstrated effective post-exposure protection against filoviruses in animal models (Dye et al., 2012; Marzi et al., 2012b; Olinger et al., 2012; Pettitt et al., 2013; Qiu et al., 2012; Qiu et al., 2014). MAbS can be produced on large scale and offer more reproducible effects than polyclonal sera from survivors. However, most MAbS available only recognize Ebola virus. Very few are yet described against Marburg virus, and no antibodies are yet described that cross-react among the filoviruses. Indeed, Marburg and Ebola GP are 72% different in protein sequence, and the filoviruses are thought to be antigenically distinct. Further, there is no structure available for the unique Marburg virus GP, by which we may interpret differences in requirements for viral entry, or develop immunotherapeutics or inhibitors of entry.

Here, we report the crystal structure of the trimeric, receptor-competent form of Marburg virus GP in complex with a neutralizing antibody, termed MR78, that was identified in a recent human survivor of Marburg virus infection (Flyak et al., 2015). Atypically, MR78 cross-reacts to cleaved Ebola virus GP. An additional structure of MR78 in complex with Ebola virus GP illustrates the basis of the cross-reactivity: the antibody binds a hydrophobic “trough” at the top of GP1, the sequence and structure of which are conserved across the filoviruses. We propose that this trough is the binding site of the critical domain C of the NPC1 receptor. Indeed, MR78 blocks binding of domain C to Marburg GP. Further, the extended third complementarity-determining region of the heavy chain (CDR H3) of MR78 mimics the glycan cap that shields this site on Ebola virus prior to entry and may mimic the receptor itself. These crystal structures plus additional biophysical analysis of complete, mucin-containing Ebola and Marburg GP ectodomains reveal that the receptor-binding site is masked on the surface of Ebola virus but more exposed on the surface of Marburg virus. These findings may explain why a cross-reactive antibody such as MR78 has not been identified in studies of Ebola virus.

RESULTS

Structure Determination

Trimeric GP ectodomains for Marburg virus (MARV; strain Ravn) or Ebola virus (EBOV, also known as Ebola Zaire; strain Mayinga) were expressed in Drosophila S2 cells, with or without their mucin-like domains (GP and GPαmuc, respectively). MARV and EBOV GPαmuc were further proteolyzed by trypsin or thermolysin, respectively, to produce cleaved GP (GPcl) resembling the version of GP competent for receptor binding in the endosome (Figure S1A). Three hundred versions of MARV GP were engineered and complexed with 22 different MAbS in order to find a crystallizable combination. Hundreds of crystals of the final MARV GPcl-MR78 combination were grown and screened for X-ray diffraction: just one crystal yielded suitable diffraction.

Diffraction to 3.6 Å resolution was obtained from a single crystal of the MARV GPcl-Fab MR78 complex. The structure was determined by molecular replacement using EBOV GP and Fab KZ52 (Lee et al., 2008) as search models and was refined to Rwork of 24.7 % and Rfree of 27.9 % (Table S1). Four GP-Fab complexes are contained in the asymmetric unit: one complete trimer and one other monomer, which forms its biologically relevant trimer around a crystallographic 3-fold axis.

Differences in GP Structure between EBOV and MARV

Although the overall organization is similar between Marburg and Ebola GPs (1.8 Å rmsd among 212 Cα atoms) (Figures 1A and 1B), several structural differences exist that may explain their differing requirements for cellular entry. The first difference is that the intra-GP1 disulfide bond formed by C121 and C147 in ebolavirus GP structures (Ebola [Lee et al., 2008] and Sudan [Bale et al., 2012; Dias et al., 2011]) does not exist in MARV. In MARV, the two cysteines are replaced instead with L105 and H131 (Figure 1C and Figure S1B). As a result, the equivalent polypeptides, which form the crest of the receptor-binding subunit, differ in structure and flexibility. In the ebolaviruses, the polypeptide bearing C147 (residues 145 to 150) turns inward, toward the trimer center to disulfide bond to C121. In MARV, the equivalent polypeptide (residues 129 to 134) turns outward into solvent, away from the trimer center.

A second difference between MARV and the ebolaviruses lies at the base of the cathepsin cleavage loop. In MARV, these residues (172-180) form a clear alpha helix (α2), which packs against the outside of the GP2 fusion loop, interacting with both the N- and C-terminal strands of the fusion loop (Figure 1D). In ebolaviruses, the equivalent residues predict to form a loop rather than a helix and are disordered (Bale et al., 2012; Dias et al., 2011; Lee et al., 2008). In MARV GP, the peptide connecting this α2 helix to β14 in the glycan cap would necessarily and immediately cover the both N- and C-terminal arms of the GP2 fusion loop, and if uncleaved, would hinder the conformational changes of fusion. Structural differences in α2 of MARV may prevent effective processing by cathepsin B.

The third difference in the MARV GP structure lies at the N terminus, in the base of the β sheet that forms the GP1 spool, about which the metastable GP2 subunit is wound. In EBOV, the base of the spool connects to the anchoring GP1-GP2 disulfide bond by a short stretch of polypeptide that intimately interacts with GP2. This short connecting polypeptide contains an N-linked glycan at Asn40, and also contains residue Asp47, which renders EBOV dependent on cathepsin B for entry (Misasi et al., 2012). In EBOV entry, cathepsin B removes an additional and critical 1 kDa of mass from GP beyond that removed by cathepsin L, but the site and consequences of that extra cleavage event are not yet known. We propose that if cathepsin B cleaves this connecting loop, EBOV GP2 would be freed from the constraints of the disulfide bond and better able to undergo the conformational rearrangements of fusion. Our crystal structure reveals that MARV, which is cathepsin B-independent, is structured differently from EBOV at the same site. In MARV, the base of the GP1 spool is more mobile and is shifted toward the center of the trimer, inside of the fusion loop. Further, unlike in ebolaviruses (Dias et al., 2011; Bale et al., 2012), the polypeptide connection to the MARV GP1-GP2 disulfide could not be visualized and the N-linked glycan is absent. The nearest glycan is instead attached to residue 171 on the MARV GP1 β sheet itself (Figure 1E). These differences in sequence, glycosylation, mobility, and conformation likely allow MARV to be cleaved by other enzymes and render MARV cathepsin B-independent.
Overall Organization of the MARV or EBOV GPcl Bound to Fab MR78

The crystal structure of MARV GPcl in complex with the Fab fragment of MR78 indicates that MR78 binds the membrane-distal head of GP1 (Figure 2A). We determined an additional, low-resolution structure of EBOV GPcl bound to both MR78 and KZ52. The ternary EBOV complex, determined by molecular replacement, demonstrates that the MR78 antibody recognizes a similar site on both MARV and EBOV (Figures 2B, S2, and Table S1). MR78 binds into a highly conserved hydrophobic trough revealed at the top of the EBOV GP1 core, after removal of the glycan cap by proteolytic cleavage in the endosome. Although MARV and EBOV diverge significantly in sequence overall, residues contained in this site, the MR78 epitope, are 85% similar between the viruses (Figures 3A and S1B).

Likely Receptor-Binding Site

The location and structural conservation of this site suggest that it could be the binding site of the NPC1 receptor, used by all known filoviruses (Carette et al., 2011; Côté et al., 2011; Miller et al., 2012; Ng et al., 2014). Indeed, in ELISA, MR78 inhibits binding of NPC1 domain C to MARV GP (Figure S3A). This site, at the apex of cleaved GP1, resembles an ocean wave morphology, with a lower trough beneath a rising crest. The trough is hydrophobic and is formed by α1, β4 and the loop that connects them (residues 63-74 in MARV). It is 22 Å wide and 8 Å deep at F72. The crest is hydrophilic, includes charged residues previously identified as essential for virus entry (Dube et al., 2009; Manicassamy et al., 2005; Manicassamy et al., 2007), and is formed by strands β7, β9 and their connecting loops (residues 92–106 and 120–134 in MARV). The 120–134 loop contains H131, which replaces the cysteine and the intra-GP1 disulfide bond of EBOV (Figure 3B).

Here, we show by ELISA that a Q128S and N129S double mutant in MARV GP abrogates binding to NPC1 domain C (Figure S4A). Q128 and N129 are at the tip of the crest and could make direct hydrophilic interaction with NPC1. The trough itself is formed by hydrophobic side chains, such as F72 (equivalent to F88 in EBOV). Also forming the trough are the main chains of hydrophilic residues; these polar side chains reach away from the trough into the trimer to make key stabilizing contacts to GP2. Two examples are R73 and K79, previously shown to be essential for MARV infectivity (Manicassamy et al., 2007). In the crystal structure, R73 makes multiple hydrogen bonds to the fusion loop.
of the neighboring protomer in the trimer (Figure S4B) and likely plays a key role in maintaining the prefusion structure or transmitting a conformational change to the fusion loop after receptor binding. K79 interacts with the main chain of residues 574–577 of GP2 (Figure S4C), residues that connect the separated helical segments of the first heptad repeat. We propose that binding of NPC1 domain C involves contact with the hydrophilic crest and hydrophobic trough, and that binding in the trough may transmit conformational changes to GP2 via R73 and K79 (equivalent to R89 and K95 in EBOV). Although MR78 binds both MARV and EBOV GPcl, it only outcompetes NPC1 domain C for binding of MARV GPcl (Figure S3B). MR78 may have lower affinity for EBOV GPcl than MARV GPcl or domain C may bind the GPs slightly differently.

**GP-MR78 Interactions**

The interaction surface between the MR78 antibody and MARV GP buries 976 Å² of molecular surface and is primarily hydrophobic. Contact is mediated by both the heavy and light chains, but the primary region of interaction is the 17-residue CDR H3 (Figures 3C and S3CD), which penetrates the hydrophobic trough in MARV GP1. In this interaction, F111.2 and Y112.2 of the CDR H3 interact with P63, S67, W70, F72, I95 and I125 of MARV GP (IMGT numbering, Figure 4A).

Notably, these interactions are similar to those made by the Ebola virus glycan cap, which occupies this site prior to enzymatic cleavage in the endosome. In Ebola virus, the equivalent interactions are made by F225 and Y232 of the EBOV glycan cap interacting with P80, T83, W86, F88, L111 and V141 on EBOV GP (Figure 4B). Similarity may even extend to the key domain C preserved receptor-binding site (Barbey-Martin et al., 2002; Bizebard et al., 1995; Hong et al., 2013; Lee et al., 2014; Schmidt et al., 2013; Whittle et al., 2011; Xu et al., 2013). In many cases those influenza mAbs also use Phe or Tyr aromatic residues to interact with an aromatic residue in the viral receptor binding domain, suggesting that the favorable energetics and intermolecular interactions of common aromatic molecules may constitute a canonical mode of binding of antiviral antibodies to recessed receptor-binding sites.

Although the MR78 epitope is largely conserved in sequence and structure between MARV and EBOV, it differs in its exposure at different stages of virus entry. MR78 binds MARV GP equally well whether MARV GP is in its uncleaved, viral-surface form or its cleaved, endosomal form. In contrast, MR78 does not bind uncleaved EBOV GP. It only binds the endosomal, cleaved form from which the glycan cap has been removed. Together, these results suggest that in EBOV, the glycan cap effectively blocks the MR78 epitope and putative receptor-binding site on the (uncleaved) viral surface, but that in MARV, the epitope and at least part of the receptor-binding site is fully exposed on the viral surface. Better exposure of this site may explain why antibodies against the putative receptor-binding site were elicited by MARV infection (see companion paper by Flyak et al., 2015), but seem to be more rarely elicited and have not yet been described against EBOV.

**Differences in Mucin-Like Domains between MARV and EBOV, and Possible Effect on Antibody Reactivity**

In addition to a glycan cap, the GP spike on the viral surface includes three heavily glycosylated mucin-like domains that are ~75 kDa each in mass and are predicted to have little secondary
All mucin-containing GPs thus far have been refractory to crystallization. In order to visualize the native glycoprotein ectodomain and position of the mucin-like domain relative to the receptor-binding core, we turned to Small-Angle X-ray Scattering (SAXS) in solution. SAXS data collected for mucin-containing EBOV or MARV GP trimers indicate that the mucin-like domains of both viruses are large and extend outward from the GP core. The radius of gyration, $R_G$, for mucin-deleted and mucin-containing MARV GPs are 50 and 72 Å, respectively, and maximum dimension, $D_{max}$, for mucin-deleted and mucin-containing GPs are 160 and 250 Å, respectively, indicating that the mucin-like domain of MARV widens the molecule up to 90 Å (Figures 5A and S5). The mucin-like domains of MARV are a bit larger than those of EBOV (67 Å $R_G$ and 225 Å $D_{max}$ for mucin-containing EBOV GP), consistent with their greater volume determined by SAXS (Figure S5C) and mass noted by SDS-PAGE (Figure S5D). The diagonal black line indicates the base of the trough. See also Figure S4.

The differing position of the mucin-like domains between MARV and EBOV would leave different surfaces exposed for immune recognition. The equatorial projection of the MARV mucin-like domain, for example, would leave the expected receptor-binding site at the top more accessible on MARV than EBOV, and further supports the notion that antibodies against the expected receptor-binding site would be more likely to be elicited using marburgvirus antigens than ebolavirus antigens. The accompanying paper (Flyak et al., 2015) and other immunization studies (Qiu et al., 2011; Wilson et al., 2000) support this notion.

In contrast, on EBOV, the upward projection of the mucin-like domains and the absence of mucin attached to EBOV GP2 would leave the EBOV base more exposed for antibody surveillance, compared to that of MARV. Indeed, in the accompanying paper by Flyak et al., none of the 18 neutralizing antibodies raised against MARV appear to bind the base of the MARV GP, while multiple neutralizing antibodies elicited by ebolaviruses are known to bind, or thought to bind, to the base of ebolavirus GP (Dias et al., 2011; Lee et al., 2008; Qiu et al., 2012; Murin et al., 2014) (Figure 5C).

**DISCUSSION**

In summary, the crystal structures and accompanying experiments indicate that MR78 binds a conserved site on the apex of GP1 that is available on the surface of MARV GP, but masked on EBOV GP prior to enzymatic cleavage. The epitope of MR78 likely overlaps with the receptor-binding site, and hydrophobic contacts made by CDR H3 to the hydrophobic trough may mimic...
those of the as-yet-unvisualized NPC1 domain C. MR78 does not neutralize authentic EBOV, likely because its epitope is masked on the EBOV surface by the mucin-like domain and glycan cap on the virus surface. MR78 does, however, neutralize authentic MARV (Flyak et al., 2015) and could be a valuable monoclonal antibody therapeutic against this extremely lethal virus. Importantly, no mAb therapeutic yet exists against MARV, and few mAbs are yet known against MARV from which such a therapeutic could be developed. The crystal structure of MARV GP presented here, and the highly conserved MR78 epitope, provide strategies for immunotherapy and templates for development of potentially broad-spectrum inhibitors of filovirus entry.

EXPERIMENTAL PROCEDURES

Construction, Expression, and Purification of MARV/EBOV GP

DNA encoding the MARV GPΔmuc ectodomain (residues 1–636 with a mucin deletion of residues 257–425), point mutants of MARV GPΔmuc and the EBOV GPΔmuc ectodomain (residues 1–637 with a mucin deletion of residues 314–462) were amplified by PCR using codon-optimized and whole-gene synthesized MARV or EBOV GPs as templates. Four point mutations in MARV GPΔmuc, F438L, W439A, F445Q, and F447N, on GP2, located around the furin cleavage site were found to improve the efficiency of furin cleavage. GP constructs were cloned into a derivative of the expression vector pMT. This derivative vector contains the puromycin resistant gene and a C-terminal double-strep tag sequence. Expression plasmids were transfected using Effectin (QIAGEN) into 80% confluent Drosophila Schneider S2 cells. The cells were first cultured in complete Schneider’s medium supplemented with 10% (v/v) FCS (LONZA), and were adapted to Insect Xpress medium by progressively modifying the Schneider/Insect Xpress medium ratio with 6.0 mg/ml puromycin. Large-scale expression of the MARV/EBOV GPΔmuc protein was performed using stable S2 cell lines in 2 l Erlenmeyer flask at 27.0 °C, induced with 0.5 mM CuSO4. Supernatants containing the expressed proteins were harvested 4 days after induction, and mixed with the Strept-Tactin affinity column binding buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 15 μg/ml Avidin [pH8.0]). The proteins were purified via Strept-Tactin affinity, followed by Superdex 200GL 10/300 (GE Healthcare Life Sciences) size-exclusion chromatography (S200 SEC), and were purified via Strept-Tactin affinity, followed by Superdex 200GL 10/300 (GE Healthcare Life Sciences) size-exclusion chromatography (S200 SEC).

Figure 4. Similarity in Recognition of the Putative Receptor-Binding Site by MR78 and the Ebola Virus Glycan Cap

(A) The CDR H3 of MR78 (yellow) reaches into the hydrophobic trough of GP1 (purple). F111.2 and Y112.2 of CDR H3 interact with P63, S67, W70, F72, I95, and I125 of MARV GP. (B) Similar residues of the EBOV glycan cap (light blue) bind into this trough on the surface of EBOV GP (blue), prior to enzymatic cleavage. Here, F225 and Y232 of the glycan cap interact with P80, T83, W86, F88, L111, and V141 in the trough (PDB ID; 3CSY).

Preparation and Crystallization of GP-Antibody Complexes

To mimic endosomal protease cleavage and produce MARV GPcl, MARV GPΔmuc was incubated with 0.01 mg trypsin at 37°C for 1 hr in 20 mM TBS [pH 8.0], 100 mM NaCl. The reaction was stopped using 0.5 mM 4-(2-Aminomethyl) benzene-sulfonfonyl fluoride hydrochloride (AEBSF), and the protein was purified by S200 SEC. EBOV GPΔc was produced by incubating EBOV GPΔmuc with 0.02 mg thermolysin overnight at room temperature in 20 mM TBS [pH 7.5], 100 mM NaCl, 1 mM CaCl2, and purified by S200 SEC. Hybridoma cells expressing the human MR78 antibody were generated from peripheral blood mononuclear cells (PBMCs) from a donor, who contracted MARV infection in the Python Cave in Queen Elizabeth National Park, Uganda in 2008 (see Flyak et al., 2015). MR78 was expressed in serum-free medium (Hybridoma-SFM, GibCO), and culture supernatants were centrifuged, sterile-filtered, and purified over HiTrap Protein G columns (GE Healthcare Life Sciences). Fab fragments were generated by standard papain digestion, with released Fc and undigested IgG removed by Protein A chromatography, and remaining Fab fragments further purified by MonoQ ion-exchange chromatography. For crystallization, purified MARV GPΔc was mixed with excess Fab MR78 for 2 days at 4°C. Complexes were separated from unbound Fab via S200 SEC. Crystals were grown by hanging-drop vapor diffusion at 20°C using 0.8 μl protein (13.0 mg ml⁻¹), in 20 mM Tris-HCl [pH 8.0], 100 mM NaCl) and 0.8 μl of mother liquor (100 mM NaCl, 50 mM MES [pH 6.5], 15 % PEG 4000, 0.5 % ethyl acetate). These crystals were cryoprotected with 25% glycerol plus mother liquor before flash cooling in liquid nitrogen. One crystal diffracted to a resolution of 3.6 Å. EBOV GPΔc was complexed with Fab KS2Z and MR78 and crystallized using hanging-drop vapor diffusion at 20°C with 1.0 μl of protein (6 mg/ml, 150 mM NaCl, 10 mM Tris [pH 7.5]) and 1.0 μl of mother liquor (100 mM NaAcetate [pH 4.6], 200 mM NH4SO4, 10% PEG 3350, 2% PEG 400). The crystals were then cryoprotected by washing in 100 mM NaAcetate [pH 4.6], 200 mM NH4SO4, 12% PEG 3350, 10% PEG 400, 10% ethylene glycol. Only diffraction to 8 Å was obtained, but this data permitted molecular replacement using Phaser (McCoy et al., 2007) and EBOV GP and KS2Z (Lee et al., 2008) as search models.

ACCESSION NUMBERS

Coordinates and structure factors have been deposited into the Protein Data Bank under the accession code 3X2D.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.01.041.
AUTHOR CONTRIBUTIONS

Experiments were conceived by E.O.S. with T.H., M.L.F., J.E.L., and Z.A.B. All structural and biochemical work was performed by T.H., M.L.F., Z.A.B., J.E.L., A.I.F. and analyzed by T.H., M.L.F., Z.A.B., J.E.L., A.I.F., R.M., D.K., Y.Y., M.H., J.E.C., and E.O.S. The manuscript was written by E.O.S. and T.H. All authors contributed to editing the manuscript and support the conclusions.

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Figure 5. MARV and EBOV Present Different Surfaces for Antibody Recognition

(A and B) Molecular envelopes of mucin-containing MARV and EBOV GP ectodomains determined by SAXS. Rendered Gaussian distributions of molecular envelopes are illustrated in light gray, with ribbon models of the crystallized MARV GPcl and EBOV GPΔmuc trimers to scale and overlaid for comparison. The trimers are illustrated as ribbons. Note that the glycan cap was removed from MARV GP used in crystallization in order to improve diffraction but was contained in the complete MARV GP used for SAXS. The glycan cap did not inhibit diffraction of EBOV GP and is included in the EBOV GP crystal structure. MARV GPcl is colored in purple (GP1) and gray (GP2). EBOV GPΔmuc is colored blue (GP1), white blue (GP1 glycan cap), and gray (GP2). MARV GP is drawn in two possible orientations because definitive placement of polypeptide is challenging at this resolution. In either orientation however, the mucin-like domains of MARV project sideways, equatorially or downward from the core of GP. In MARV, the mucin-like domain is attached to both GP1 and GP2. By contrast, in EBOV, the mucin-like domain is attached solely to GP1, there is no anchor at the base. Both these SAXS experiments and previous electron tomography (Tran et al., 2014) agree on the upward projection of the mucin-like domains in EBOV. See also Figure S5.

(C) Differing positions of the mucin-like domains between MARV and EBOV may lead to elicitation of different types of antibodies. The lower position and GP2 anchor of the mucin-like domain of MARV may better mask the base of GP but expose its upper surfaces, allowing antibodies like mAb MR78 to be elicited. The upward projection of the EBOV mucin-like domain and absence of any GP2 anchor, appear to better mask upper surfaces, but expose the base, allowing antibodies such as KZ52 (Lee et al., 2008), 2G4, 4G7 (Murin et al., 2014), and 16F6 (directed against Sudan ebolavirus [Dias et al., 2011; Bale et al., 2012]) to be elicited.


Chimeric Filoviruses for Identification and Characterization of Monoclonal Antibodies

Running title: Chimeric Filoviruses

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ABSTRACT

Recent experiments suggest that some glycoprotein (GP) specific monoclonal antibodies (mAbs) can protect experimental animals against the filovirus Ebola virus (EBOV). There is a need for isolation of mAbs capable of neutralizing multiple filoviruses. Antibody neutralization assays for filoviruses frequently use surrogate systems such as the rhabdovirus vesicular stomatitis Indiana virus (VSV), lentiviruses or gammaretroviruses with their envelope proteins replaced with EBOV glycoprotein (GP) or pseudotyped with EBOV GP. It is optimal for both screening and in-depth characterization of newly identified neutralizing mAbs to generate recombinant filoviruses that express a reporter fluorescent protein in order to more easily monitor and quantify the infection. Our study showed that unlike neutralization-sensitive chimeric VSV, authentic filoviruses are highly resistant to neutralization by mAbs. We used reverse genetics techniques to replace EBOV GP with its counterpart from the heterologous filoviruses Bundibugyo virus (BDBV), Sudan virus (SUDV), and even Marburg virus (MARV) and Lloviu virus (LLOV), which belong to the heterologous genera in the filovirus family. This work resulted in generation of multiple chimeric filoviruses, demonstrating the ability of filoviruses to tolerate swapping of the envelope protein. The sensitivity of chimeric filoviruses to neutralizing mAbs was similar to that of authentic biologically derived filoviruses with the same GP. Moreover, disabling the expression of the secreted GP (sGP) resulted in an increased susceptibility of an engineered virus to the BDBV52 mAb isolated from a BDBV survivor, suggesting a role for sGP in evasion of antibody neutralization in the context of a human filovirus infection.
The study demonstrated that chimeric rhabdoviruses in which G protein is replaced with filovirus GP, widely used as surrogate targets for characterization of filovirus neutralizing antibodies, do not accurately predict the ability of antibodies to neutralize authentic filoviruses, which appeared to be resistant to neutralization. However, a recombinant EBOV expressing a fluorescent protein tolerated swapping of GP with counterparts from heterologous filoviruses, allowing high-throughput screening of B cell lines to isolate mAbs of any filovirus specificity. Human mAb BDBV52, that was isolated from a survivor of BDBV infection, was capable of partially neutralizing a chimeric EBOV carrying BDBV GP in which expression of sGP was disabled. In contrast, the parental virus expressing sGP was resistant to the mAb. Thus, the ability of filoviruses to tolerate swapping of GP can be used for identification of neutralizing mAbs specific to any filovirus and for characterization of mAb specificity and mechanism of action.
The family Filoviridae is composed of the genus Ebolavirus, which includes Ebola (EBOV), Sudan (SUDV), Taï Forest (TAFV), Reston (RESTV), and Bundibugyo (BDBV) viruses, the genus Marburgvirus, which includes Marburg (MARV) and Ravn (RAVV) viruses, and the putative genus Cuevavirus, which includes Lloviu virus (LLOV) (1). All of these viruses, with the exception of TAFV, RESTV and LLOV, are known to cause disease outbreaks in humans with high case fatality (2, 3). The recent outbreak of EBOV disease in Western Africa (4) demonstrated that filoviruses can cause large epidemics. In addition, identification of the “new” filoviruses BDBV and LLOV was reported as recently as in 2007 or 2011, respectively (5, 6), suggesting the possibility of emergence or identification of previously unknown filoviruses.

For decades, no treatment demonstrated protective efficacy against filoviruses in the non-human primate model, which is considered the best model of filovirus disease predictive for a similar effect in humans. However, recently developed treatments based on polyclonal (7) or monoclonal antibodies (mAbs) (8-10) have shown impressive levels of efficacy in non-human primates. Development of mAb-based treatments and understanding of mechanisms of antibody neutralization of RNA viruses can be greatly facilitated by development of reverse genetics systems, which allow recovery of recombinant viruses from DNA copies of their genomes or antigenomes. The advantages of such systems for work with polyclonal or monoclonal Abs include the possibility of introduction of mutations in genes encoding major protective antigens, such as the glycoprotein (GP), which is the sole envelope protein of filoviruses. GP is expressed as a precursor protein that is cleaved post-translationally to GP1 and GP2 subunits, and the mature integral membrane protein is present on the surface of viral particles as two disulfide-linked subunits (11, 12). The GP gene of ebolaviruses encodes two proteins: the full-length GP, which is a part of the viral particles and a type I transmembrane protein, and the secreted GP (sGP). It also encodes a much less abundant small soluble GP (ssGP). The GP gene does not have a continuous open reading frame, and thus the expression of full-length GP and ssGP result from transcriptional editing. In contrast, sGP does not require transcriptional editing, and has an identical N-terminal part of GP, but a unique C-terminal part (13-15). Unlike ebolaviruses, marburgvirus GP genes have a
continuous open reading frame that encodes full-length GP but not sGP (16, 17). Another advantage of reverse genetics techniques is the possibility to engineer viruses expressing green fluorescent protein (GFP) or another reporter protein to visualize infection. However, development of reverse genetics systems is very labor and time consuming and is not always successful. To date, such systems have been developed only for three filoviruses: EBOV, MARV and RESTV (reviewed in reference 18), and are not available for the other filoviruses causing a severe disease, including SUDV, BDBV or RAVV. In the absence of reverse genetics systems and/or biosafety level 4 (BSL-4) facilities required for work with filoviruses, researchers use various surrogate systems for characterization of filovirus-specific mAbs or investigation of various steps of filovirus life cycle involving GP. These systems include chimeric vesicular stomatitis Indiana viruses (VSV) with the G protein replaced with filovirus GP (19-21), and pseudotyped gammaretroviruses (22, 23) and lentiviruses (24-26), which have their respective envelope proteins replaced with a filovirus GP provided in trans. It is generally assumed that these surrogate systems can be used to accurately characterize neutralizing properties of filovirus polyclonal or monoclonal Abs.

In the present study, we used the EBOV reverse genetics system to show that the virus can tolerate replacement of GP with its counterpart not only from heterologous ebolaviruses, but remarkably also from a more distantly related marburgvirus and cuevavirus. The generated chimeric viruses were used, in parallel, with chimeric VSVs, for characterization of filovirus-reactive mAbs isolated from human survivors of previous BDBV or MARV infection. We show here that filoviruses, including the chimeric filoviruses, are more resistant to neutralization by many mAbs, when compared to their chimeric VSV counterparts. These data suggest that chimeric VSVs may not be optimal for accurate quantification of neutralizing activity of filovirus antibodies. We also demonstrate the development of a high-throughput system suitable for functional screening and analysis of large panels of filovirus mAbs. Finally, we used the chimeric viruses to demonstrate that BDBV sGP serves as a decoy for a BDBV GP-specific antibody isolated from a BDBV survivor.
MATERIALS AND METHODS

Construction of chimeric EBOV viruses and biological filovirus isolates used in the study. To construct the full-length genome cDNA of EBOV with its GP swapped with that of BDBV, SUDV or MARV, we used the plasmid carrying the genomic RNA of wild-type EBOV (pEBOwt) and its modified version with the transcriptional cassette encoding enhanced green fluorescent protein added between the NP and VP35 genes (pEBO-eGFP) (27), which were provided by Dr. Jonathan Towner and Dr. Stuart Nichol (CDC). First, the pEBOwtΔBamHI-SbfI,Ascl-PspOMI plasmid subclone was generated from the pEBOwt construct by consecutive two-step removal of BamHI-SbfI and Ascl-PspOMI fragments; after pEBOwt digestion with the each pair of restriction endonucleases, the residual vector part was treated with the Klenow fragment of DNA polymerase I and self-ligated. The resulting subclone was subjected to mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) for introduction of NheI and XhoI restriction endonuclease sites flanking the GP open reading frame (ORF). Additionally, the similar pEBOwtΔBamHI-SbfI,Ascl-PspOMI subclone was generated with BamHI restriction endonuclease site instead of XhoI downstream of the EBOV GP ectodomain encoding region corresponding to the genomic sequence of EBOV Ebola virus/H.sapiens-tc/COD/1976/Yambuku-Mayinga, nucleotides 6039-7987 (GenBank accession number NC_002549.1). For cloning of GP ORF of MARV, BDBV and SUDV, Vero-E6 cells were infected with Marburg virus/H.sapiens-tc/UGA/2007/Kitaka-200702854, Bundibugyo virus/H.sapiens-tc/UGA/200706291/Butalya-811249, or Sudan virus/H.sapiens-tc/UGA/2000/Gulu-200011676, and subjected to RNA isolation following cell lysis in TRIzol reagent (LifeTechnologies, Carlsbad, CA). Total RNA was reverse-transcribed, and PCR fragments corresponding to GP ORFs of three different filovirus species were generated and cloned into pUC19 vector by SphI and Xmal sites. In order to perform subsequent cloning steps toward generation of full-length clones, the following restriction endonuclease sites were knocked down by introduction of silent mutations: BamHI and XhoI in MARV GP ORF, Apal and Nhel in BDBV GP ORF, and Apal, Sacl and XhoI in SUDV GP ORF. To disable the expression of sGP, the GP gene transcriptional editing site was modified from AAAAAAAA to AAGAAGAA (antigenome DNA sense) by site-directed mutagenesis, resulting in a plasmid designated pUC19-BDBV-GPΔsGP. Next, the resulting pUC19 clones were used to PCR amplify BDBV GP
ORF with the following primers: direct,
ACAGTAGCTAGCAACACAATGGTTACATCAGGAATTCTACAATTGCCC, reverse,
ACAGTACTCGAGAAAAATTAGTAGAAATTGTGAAATACACAGCAGTG;
SUDV GP was amplified with the primers: direct, ACAGTAGCTAGCAACACAATGGGGGGTCTTAGCCTACT,
reverse, ACAGTACTCGAGAAAAATCGAAACAGCAGCTTGCAAA;
MARV GP was amplified with the primers: direct,
TCTAGCAGCTAGCAACACAATGAGGACTACATGCTTCTT, reverse,
TCTAGCAGCTCAGAAAAACTATCCATATATTGAAATAGATACGACAA; and MARV GP ectodomain was
amplified with the primers: direct, TCTAGCAGGCTAGCAACACAATGAGGACTACATGCTTCTT, and reverse,
AGTCACGTGGATCCA
GTCGGATGTCCACCATTTACCACC (NheI, XhoI or BamHI restriction endonuclease
sites are underlined, the start of GP ORF direct sequence and the end of GP ORF or GP ectodomain
complementary sequences are italicized). The PCR products were used to replace EBOV GP ORF in
pEBOwtΔBamHI-SbfI,Ascl-PspOMI subclone with ORF for GP of BDBV, SUDV or MARV by NheI and XhoI
sites, or for the replacement of EBOV GP ectodomain encoding region with that of MARV by NheI and BamHI
sites, respectively. To generate the final full-length constructs, the Apal-Sacl fragments of the generated
subclones were transferred to pEBO-eGFP plasmid for substitution of the existing EBOV GP ORF with an ORF
encoding the GP of BDBV, SUDV, or MARV, or to replace the EBOV GP ectodomain with a MARV GP
ectodomain. For the construction of EBOV full-length clone with its GP replaced by that of LLOV, we first
changed editing site in LLOV GP ORF from 8A to 7A (antigenome DNA sense) by mutagenizing pBsII SK (+)-
LLOV GP plasmid (provided by Dr. Ayato Takada, reference 28 ) to make it identical to the original LLOV
sequence (6). Also, two existing KpnI restriction endonuclease sites in LLOV GP ORF were knocked down by
introduction of silent mutations. The resulting LLOV GP ORF cDNA was PCR-amplified using the following
primers: direct, TCTAGCAGGCTAGCAACACAATGAGGACTACATGCTTCTT, and reverse,
TCTAGCAGCTCAGAAAAATCATCGTGTATTCTGCACA (NheI or XhoI restriction endonuclease sites are
underlined, the start of LLOV GP ORF direct sequence and the end of LLOV GP ORF complementary
sequence are italicized), and cloned into pEBOwtΔBamHI-SbfI,Ascl-PspOMI plasmid. The Apal-KpnI fragment
from the resulting subclone was transferred to the pEBO-eGFP full-length clone with one of its KpnI sites (in
polymerase L ORF, nucleotides 14292-14297 in EBOV genome) disabled by introduction of a silent mutation for substitution of the existing ORF of EBOV GP with an ORF encoding the GP of LLOV. The chimeric viruses Ebola virus/H.sapiens-rec/COD/1976/Yambuku-Mayinga-eGFP-BDBV_GP (hereafter referred as EBOV/BDBV-GP), its derivative Ebola virus/H.sapiens-rec/COD/1976/Yambuku-Mayinga-eGFP-BDBV_GPdelta_sGP (referred as EBOV/BDBV-GPΔsGP) that is deficient in the production of sGP, Ebola virus/H.sapiens-rec/COD/1976/Yambuku-Mayinga-eGFP-SUDV_GP (referred as EBOV/SUDV-GP), Ebola virus/H.sapiens-rec/COD/1976/Yambuku-Mayinga-eGFP-MARV_GP (referred as EBOV/MARV-GP), Ebola virus/H.sapiens-rec/COD/1976/Yambuku-Mayinga-eGFP-MARV_GPed (referred as EBOV/MARV-GPed), and Ebola virus/H.sapiens-rec/COD/1976/Yambuku-Mayinga-eGFP-LLOV_GP (referred as EBOV/LLOV-GP) were rescued as previously described (29) and propagated by two passages in Vero-E6 cell culture monolayers. Genomic RNA of all recovered viruses was sequenced using Illumina HiSeq 1000 sequencing system as previously described (30) and the 3’ and 5’ termini were sequenced by RNA circularization as previously described (31). The sequences were deposited in GenBank, accession numbers KU174137-KU174142. Work with the filovirus full-length clones was performed in a laboratory approved by the NIH Recombinant DNA Advisory Committee. Generation of the chimeric viruses was approved by the UTMB Institutional Biosafety Committee. Recovery of the recombinant filoviruses and all work with filoviruses were performed in the BSL-4 facility of the Galveston National Laboratory. The growth kinetics experiments on chimeric EBOV viruses were performed as previously described (29). BDBV and MARV were provided originally by the Special Pathogens Branch of the U.S. Centers for Disease Control and Prevention (CDC) and deposited at the World Reference Center of Emerging Viruses and Arboviruses (WRCEVA) housed at the Galveston National Laboratory, the University of Texas Medical Branch at Galveston. BDBV isolate 200706291 Uganda was isolated originally from the serum of a patient during the first recorded outbreak caused by this virus (5), and passaged three times in Vero-E6 cells. MARV isolate 200702854 Uganda was isolated originally from a subject designated “Patient A” during the outbreak in Uganda in 2007 (32, 33) and underwent four passages in Vero-E6 cells.

Immunostaining of chimeric EBOV plaques. Vero-E6 cell culture monolayers were inoculated with dilutions of chimeric EBOV constructs, covered with 0.9% methylcellulose (Sigma-Aldrich, Saint Louis, MO), and
incubated at 37°C. At day 6 after infection, the overlay was removed, cells were fixed with formalin for 24 hours, taken out of BSL-4, and blocked in 5% skim milk in phosphate buffered saline (PBS) containing 0.1% Tween-20 (Sigma-Aldrich) for one hour. Next, monolayers were incubated for 1 hour at 37°C with the selected mAbs (1 µg/ml) in solution, or with 1:1,000 dilution of rabbit polyclonal serum raised against EBOV isolate Mayinga or MARV isolate Musoke, and then washed three times with blocking solution. Thereafter, the respective goat anti-human or goat anti-rabbit IgG antibodies conjugated with horse radish peroxidase (KPL, Gaithersburg, MD) were added at 1:1,000 dilution in blocking buffer, and monolayers were incubated for one hour at 37°C and washed three times with PBS. Virus plaques were visualized by staining with the 4CN two-component peroxidase substrate system (KPL, Gaithersburg, MD).

Chimeric VSV with filovirus GP proteins. VSV/MARV-GP was constructed and recovered as previously described (34). VSV/BDBV-GP was provided by Dr. Thomas Geisbert (University of Texas Medical Branch at Galveston, Galveston, Texas), and VSV/EBOV-GP (35) was provided by Dr. Heinz Feldmann (Rocky Mountain Laboratories, National Institutes of Health, Hamilton, Montana).

Plaque reduction assay. For plaque-based neutralization assays, 150 PFUs of filoviruses or chimeric VSVs were pre-incubated with varying concentrations of mAbs in 100 µl volume for 1 hour at 37°C in triplicate and placed on monolayers of Vero-E6 cells in 24-well plates. After adsorption of virus for 1 hour at 37°C, cells were overlaid with 1 ml of 0.9% methylcellulose in MEM medium containing 10% of fetal bovine serum (Quality Biologicals, Gaithersburg, MD) and 0.1% of gentamicin sulfate (Mediatech, Manassas, VA) and incubated at 37°C. Chimeric VSV plaques were visualized by staining monolayers with 0.25% crystal violet solution in formalin on day 3, 4 or 5 after infection, and filovirus plaques were immunostained with rabbit anti-EBOV polyclonal serum on day 6 after infection as described above. Plaques were counted, and neutralization curves were plotted as percentages of reduction of plaque numbers as compared to mock-neutralized virus.

High-throughput screening neutralization assay. Four hundred PFUs of recombinant EBOV expressing eGFP or EBOV/BDBV-GP were incubated with varying concentrations of mAbs in black polystyrene 96-well
plates with clear bottoms (Corning, NY) for 1 hour at 37°C in MEM medium containing 10% fetal bovine serum and 0.1% of gentamicin sulfate. Next, 4 x 10^4 Vero-E6 cells in the same medium were added to the virus-antibody mixtures and incubated at 37°C for 4 days. The fluorescence intensity of infected cells at 488 nm wave length was measured in triplicate using the 2104 EnVision multilabel reader (PerkinElmer, Waltham, MA). The signal readout was normalized to virus control aliquots with no mAb added and presented as the percentage of neutralization.

**Binding of antibodies to the recombinant GPs of various filovirus species.** Soluble forms of GP of BDBV, EBOV, SUDV, MARV or sGP of BDBV, EBOV, SUDV were coated overnight onto 384-well plates (Thermo Scientific Nunc, Waltham, MA) at 1 μg/ml in PBS. Plates were blocked with 25 µl of blocking solution/well for 1 hour. Blocking solution consisted of 10 g powdered milk (Bio-Rad, Hercules, CA), 10 ml of goat serum (Gibco, Waltham, MA), 100 ml of 10x PBS, and 0.5 ml of Tween-20 mixed to a 1 l final volume with distilled water. Purified antibodies were applied to the plates at a concentration 10 μg/ml in blocking solution for 2 hr. The presence of antibodies bound to the GP and sGP proteins was determined using goat anti-human IgG alkaline phosphatase conjugate (Meridian Life Science, Memphis, TN) and p-nitrophenol phosphate substrate tablets (Sigma-Aldrich), with optical density read at 405 nm after 1 hour.

**RESULTS**

*Filoviruses are resistant to antibody neutralization compared to chimeric VSVs.* We used human Abs to compare their ability to neutralize filoviruses EBOV or MARV on the one hand or recombinant VSVs expressing the same filovirus GPs on the other hand (Fig. 1A). We first tested a recombinant form of the EBOV-specific antibody KZ52, isolated from a phage display library constructed from antibody genes from a human survivor of natural EBOV infection (36), which is protective in the guinea pig model of EBOV infection (37). The antibody appeared to neutralize authentic EBOV less efficiently than VSV/EBOV-GP. Next,
antibodies BDBV43 and BDBV52, isolated from a human survivor of BDBV infection (38) were tested. Again, authentic BDBV was less efficiently neutralized than VSV/BDBV-GP. Moreover, BDBV52 did not neutralize authentic BDBV at any concentration tested (up to 200 µg/ml), but effectively neutralized VSV/BDBV-GP. Analysis of neutralization by additional BDBV antibodies from the survivor suggested a similar pattern, i.e., less efficient neutralization of authentic as compared to VSV/BDBV-GP (data not shown). Analysis of the antibody MR201 that we isolated previously from a MARV survivor (34) also demonstrated a great difference between the neutralization of authentic MARV and VSV/MARV-GP. For example, 50 µg/ml of MR201 neutralized only 7% of MARV, but as much as 94% of VSV/MARV-GP. Taken together, these data suggest that authentic filoviruses generally are more resistant to neutralization by mAbs compared to chimeric VSVs, which can, as a result of these findings, produce greatly exaggerated data on mAb neutralization.

Replacement of the EBOV GP with its counterpart from heterologous ebolaviruses results in viable chimeric viruses. Infection of cells with the recombinant EBOV expressing eGFP from an added gene results in a bright eGFP fluorescence in infected cells starting at 36-48 hrs post-infection (27), which opens the possibility for use of this virus for development of a high-throughput screening of mAbs. As noted above, the genus *Ebolavirus* includes five virus species whose representatives EBOV, BDBV and SUDV cause a severe disease in humans. We therefore attempted to generate recombinant replication-competent EBOV-derived viruses in which the GP protein was exchanged with that of BDBV or SUDV. We replaced the ORF of EBOV GP in the EBOV full-length clone with that of BDBV or SUDV and used the plasmids in virus recovery experiments, as previously described (27, 29) (Fig. 2A,D). Indeed, the experiments resulted in recovery of viable chimeric viruses expressing eGFP: EBOV/BDBV-GP and EBOV/SUDV-GP (Fig. 3A); sequencing analysis revealed no adventitious mutations in the GP gene or elsewhere in the genome. We then compared the multi-step growth kinetics of the recombinant viruses (Fig. 3B). Contrary to our expectations, EBOV/BDBV-GP replicated slightly faster than EBOV, and EBOV/SUDV-GP replicated much faster than EBOV (Fig. 3B). For example, on day 1 or 2, the respective titers of EBOV/SUDV-GP were greater than those of EBOV by 2.5 or 1.3 log₁₀, although the final viral titers of all viruses were comparable. These data suggest that EBOV can tolerate swapping of GP with its counterparts from heterologous ebolaviruses.
**EBOV can tolerate swapping of the glycoprotein with that of MARV and LLOV.** The two members of genus *Marburgvirus*, MARV and RAVV, have only partial antigenic relatedness (39), further suggesting the need for development of a single filovirus platform capable of expressing the GPs from individual marburgviruses for accurate characterization of antibodies. Filovirus GP is a type I transmembrane protein (16, 17), which interacts with VP40 (40); its transmembrane domain affects conformation of the protein (41) and is required for incorporation in viral particles (42). Since the MARV GP cytoplasmic tail (amino acids RIFTKVIG) has no similarity to its EBOV GP counterpart (amino acids KFVF), and the transmembrane domains exhibit only a limited similarity (16, 43), the compatibility between MARV GP with EBOV particles was difficult to predict. We therefore initially attempted to replace the EBOV GP ectodomain only (which represents the GP lacking the cytoplasmic tail and the highly conserved transmembrane domain) with that of MARV (Fig. 2B,D). As a result, a fully viable virus designated EBOV/MARV-GPed was recovered (Fig. 3A), which replicated at a rate similar to that of EBOV (Fig. 3B). Since EBOV easily tolerated swapping of the GP ectodomain, we next attempted to replace the whole GP with that of MARV (Fig. 2B). This approach also resulted in recovery of a viable virus (Fig. 3A) that replicated at a level similar to that of EBOV (Fig. 3B), suggesting that the interaction of the GP cytoplasmic tail with VP40 (the matrix protein) may be not highly specific. We also attempted to replace the whole GP with its counterpart from cuevavirus LLOV, whose RNA was identified in a dead bat in Spain (6) but the virus was never isolated. This experiment resulted in recovery of a viable virus designated EBOV/LLOV-GP (Fig. 2A,C), whose replication was slightly reduced as compared to EBOV (Fig. 3B). Again, adventitious mutations were not detected in GP or elsewhere in the genome of EBOV/MARV-GP, EBOV/MARV-GPed and EBOV/LLOV-GP. Thus, EBOV can tolerate swapping GP with its counterparts not only from heterologous ebolaviruses, but also from more distantly related and distinct marburgvirus and cuevavirus.

*The generated chimeric filoviruses produce plaques, which can be stained by mAbs specific to their glycoproteins.* We next tested the ability of mAbs isolated from filovirus survivors to bind to plaques formed by the constructed viruses. We tested the binding of mAbs BDBV43 or BDBV52 recently isolated from a survivor...
of BDBV infection (A.I.F. et al., unpublished data) and MR78, MR235 or MR246 from a survivor of MARV 
infection (34) (Fig. 4A). The comparison was performed in parallel using rabbit hyperimmune serum against 
EBOV or MARV. We found that rabbit EBOV-specific immune serum was able to stain plaques not only for 
EBOV, but also for all constructs generated, including EBOV/MARV-GP and EBOV/MARV-GPed. This 
observation can be explained by the contribution of binding of antibodies in the immune serum that recognize 
internal EBOV proteins, such as NP and VP40. Interestingly, BDBV43 stained the three ebolavirus GP-based 
constructs with varying intensity and also EBOV/MARV-GP and EBOV/MARV-GPed, although with a low 
intensity, while BDBV52 stained EBOV/BDBV-GP and weakly EBOV/MARV-GP and EBOV/MARV-GPed, but 
not EBOV and EBOV/SUDV-GP. These data suggest the two BDBV mAbs interact with epitopes that are 
partially conserved across all or some members of the family Filoviridae. In contrast, MARV polyclonal 
antibodies stained plaques of EBOV/MARV-GP or EBOV/MARV-GPed but not the ebolavirus constructs. 
Again, similarly to polyclonal MARV antibodies, the three MARV mAbs stained only MARV, but not the 
ebolavirus GP-based constructs. The GP proteins of EBOV and MARV have considerable amino acid similarity 
(44), with several stretches of four or more identical amino acids located in the receptor-binding region of GP1 
and heptad repeats 1 and 2 in GP2 (based on comparison of EBOV isolate Mayinga and MARV isolate 
Uganda, Fig. 4B). In contrast, the mucin-like domains of EBOV and MARV have almost no sequence similarity. 
Therefore, the two BDBV mAbs react with conserved epitopes, while the two MARV-specific mAbs and the 
polyclonal MARV Abs are directed against more variable epitopes. These data demonstrate that chimeric 
filovirus constructs are useful in the characterization of antibodies specific for any filovirus species.

**Chimeric filoviruses are neutralized according to the GP ectodomain specificity of mAbs.** To determine 
the binding specificities of the mAbs used in the study, we tested a panel of mAbs for their ability to bind a 
panel of recombinant filovirus GP or sGP proteins (Fig. 5A). All mAbs tested, with the exception of BDBV43, 
bound exclusively to full-length GP of the targeted filoviruses, and all of the mAbs except BDBV43 and 
BDBV52 did not detectably bind to sGP. In contrast, BDBV43 bound to both GP and sGP of all three 
ebolaviruses: EBOV, BDBV and SUDV, but not to MARV, while BDBV52 bound to both GP and sGP of BDBV 
only. We next tested the ability of selected mAbs to neutralize EBOV, BDBV, MARV, EBOV/MARV-GP or
EBOV/MARV-GPed (Fig. 5B). As expected, EBOV was neutralized effectively by mAb KZ52 and, to a lesser
degree, by BDBV43, but not by BDBV41 or BDBV52. BDBV was neutralized by BDBV41 and BDBV43, but not
by BDBV52, despite the fact that this mAb binds to BDBV GP. MARV and the chimeric viruses carrying MARV
GP or its ectodomain were neutralized by MR78, but not the other mAbs. Thus, the GP ectodomain
specificities of chimeric filoviruses determine the neutralization efficiencies of antibodies, which do not
necessary correlate with the protein binding data. These data suggest that chimeric filoviruses are useful for
highly specific antibody neutralization tests.

**Use of the chimeric filoviruses for a high-throughput screening (HTS) assay of mAbs.** Neutralization
tests of large panels of mAbs or multiple serum samples against multiple filovirus species by conventional
plaque reduction assay, which must be performed in BSL-4 biocontainment, is laborious. We therefore tested
the generated chimeric viruses as targets for a HTS neutralization assay in which eGFP fluorescence provided
a measure of remaining infectivity and thus a measure of antibody neutralization. In preliminary experiments,
we performed test neutralizations of varying doses of EBOV/BDBV-GP ranging from 4 to 4,000 PFUs with
varying concentrations of mAb BDBV41. Following a one hour incubation, virus-antibody aliquots were mixed
with varying amounts of Vero-E6 cells ranging from 2,500 to 40,000, and then added as a suspension to
individual wells of 96-well plates. eGFP fluorescence was read on days 2, 3, 4, 5 and 6. As an example of our
preliminary data, infection of 40,000 cells with 150 or 300 PFUs resulted in a two-fold difference in the signal
on day 4 (Fig. 6A). In another example, residual infectivity in Vero-E6 cells was measured in a broad range of
various concentrations of mAb BDBV41 mixed with EBOV/BDBV-GP in triple aliquots. Quantitation of eGFP
signal on a fluorescence plate reader on day 4 post infection demonstrated that the level of eGFP signal was
inversely proportional to the amount of mAb added (Fig. 6B). Interestingly, based on visual examination of
plates, this antibody inhibited or prevented spread of the viral infection, but did not completely eliminate initial
infectious foci even at the greatest concentration tested, 200 µg/ml (Fig. 6C). Based on the optimization
experiments, we found that inoculation of 40,000 cells with 400 PFU (MOI of 0.01) of EBOV/BDBV-GP gave
the greatest possible dynamic range of the signal at various antibody concentrations (data not shown). Next,
we used the optimized assay conditions to determine neutralization potency of BDBV41 in comparison with the
classic plaque reduction assay with BDBV, VSV/BDBV-GP or EBOV/BDBV-GP (Fig. 6D). As in the case of KZ52, MR201 and BDBV43 (Fig. 1), VSV/BDBV-GP was more easily neutralized by BDBV41 in classic plaque reduction assays. Use of EBOV/BDBV-GP instead of BDBV for the plaque reduction assay resulted in a similar neutralization curve, and use of EBOV/BDBV-GP in a HTS assay also resulted in a neutralization curve similar to that generated by plaque reduction assays with BDBV or EBOV/BDBV-GP. Thus, chimeric filoviruses expressing eGFP can be used as a substitute for their natural counterparts in plaque reduction assays as well as being used in HTS assays to rapidly identify mAbs neutralizing individual filovirus species.

The sGP protein prevents virus neutralization by BDBV52. We next tested the possibility that the generated chimeric viruses are useful for a qualitative characterization of mAbs. We previously demonstrated that the secreted G protein of respiratory syncytial virus, which, similarly to EBOV is a non-segmented negative strand virus, reduces the efficiency of virus neutralization by serving as a decoy for neutralizing antibodies (45). More recently, a similar immune evasion mechanism was demonstrated for EBOV sGP (26). However, these studies involved mouse polyclonal antibodies, and the BDBV sGP study involved a chimeric VSV expressing EBOV GP, and therefore, the importance of this mechanism for pathogenesis of human disease remained unknown. Data presented here show that BDBV52 bound to both BDBV sGP and GP (Fig. 5A). We hypothesized that sGP serves as a decoy for BDBV52 and antibodies with similar epitope specificities, thereby preventing their ability to effectively neutralize BDBV. To test the hypothesis, we modified the GP gene of EBOV/BDBV-GP cDNA to disable the expression of sGP by mutating the transcription-editing site from UUUUUUU to UUCUUCUU (negative-sense RNA strand). As a result, the modified GP gene had the continuous open reading frame encoding GP only. The resulting virus designated EBOV/BDBV-GPΔsGP was recovered and sequencing data confirmed the stability of the mutation and lack of any adventitious mutations in the genome (data not shown). sGP is not required for viral replication in cultured cells. To determine if disabling of expression of sGP makes the virus more sensitive to neutralization by BDBV52, we compared susceptibility of the two viruses to the antibody. We found that while EBOV/BDBV-GP was completely resistant to the antibody, EBOV/BDBV-GPΔsGP was partially neutralized at the highest antibody concentration tested, 200 µg/ml (Fig. 7A), suggesting that BDBV52 binds part of sGP shared with GP (Fig. 7B). These data
demonstrate for the first time the ability of an ebolavirus to evade neutralization by a naturally-occurring human mAb isolated from a survivor.

**DISCUSSION**

The recent devastating outbreak of EBOV in Western Africa (4) demonstrated the pressing need in development of means of treatments and prophylaxes of infections caused by filoviruses. To date, the greatest progress has been achieved, which require detailed characterization of monoclonal or polyclonal Abs. Data presented in this study suggest that surrogate systems such as chimeric VSV expressing filovirus GP may produce misleading results and therefore appear to be suboptimal for reliable characterization of filovirus antibodies. The simultaneous circulation of multiple lineages of filoviruses in the same outbreak (46, 47) along with the emergence of “new” filoviruses (5, 6), and improved methods for isolation of mAbs from survivors (34, 38, 48) along with the utility of their use in therapeutics strongly support the need for improved efficient means of rapid screening and characterization of large panels of mAbs. While a previous study demonstrated the possibility of generation of viable ebolaviruses with some genes replaced with counterparts from a heterologous ebolavirus (49), this study demonstrates that EBOV can easily tolerate exchange of GP not only from ebolaviruses, but also from more distantly related marburgvirus and cuevavirus. Each of the chimeric viruses easily tolerated and expressed eGFP from an added gene. Moreover, we show that chimeric filoviruses are useful for a HTS screening process to identify neutralizing mAbs. In addition, we show that binding to GP does not necessarily predict the ability of a mAb to neutralize a filovirus, the effect most likely related to a different conformation of GP in a free form and in a viral particle. Taken together, these results illustrate the practicality of a quick exchange of GP in EBOV with its counterpart from any circulating filovirus and the use of the resulting chimeric filoviruses for a rapid analysis of large panels of monoclonal or polyclonal Abs.

The present study shows that filoviruses are more resistant to neutralization by mAbs than chimeric VSV with filovirus GP widely used for quantitative analysis of filovirus-specific mAbs. While the exact reason for this
phenomenon requires additional studies, we hypothesize that it may be related to a much greater length of filovirus particles, 1,028 nm for EBOV or 876 nm for MARV (50), as compared to 175 nm for VSV (51). The greater length of filovirus particles suggests a greater number of GP trimers per filovirus particle, which require a greater number of bound mAbs to abrogate infectivity, as compared to a chimeric VSV. However, this model cannot explain why for some antibodies, such as KZ52 and BDBV43, the difference between neutralization of a filovirus and the corresponding chimeric VSV is moderate, while for others, such as BDBV52 and MR201, the difference is dramatic (Fig. 1A). Most likely, other factors, such as epitope specificity of mAbs, also affect the observed difference.

A recently published study demonstrated that sGP interferes with the antibody-mediated neutralization of EBOV GP-lentivirus pseudotype by antisera from sGP or GP immunized mice (26). The importance of this mechanism in the context of human filovirus infections remains unclear. In a separate study we isolated BDBV52, an antibody from a survivor, which binds both the recombinant GP and sGP, but does not neutralize EBOV/BDBV-GP (38). We hypothesized that this mAb can neutralize virus in the absence of sGP. To test the hypothesis, we generated the derivative of EBOV/BDBV-GP, which does not express sGP, EBOV/BDBV-GPΔsGP. Indeed, this virus was partially neutralized by BDBV52. These data represent the first demonstration that ebolavirus survivors have mAbs that bind sGP, which do not neutralize the virus, but can partially neutralize the virus when sGP expression is disabled. Thus, expression of sGP might help the virus to evade effective antibody neutralization. Of note, a recent study demonstrated that disabling of the transcriptional editing site of the GP gene reduces EBOV virulence, but the expression of sGP per se did not affect it (52). We are unaware of any comparison of sGP expression by the various ebolaviruses; however, the identical GP transcription editing sites and the high levels of similarity of the polymerase (L) genes of EBOV, BDBV and SUDV (5, 41) suggest that the levels of expression of sGP by these viruses and by EBOV/BDBV-GP and EBOV/SUDV-GP are comparable.

In summary, these data suggest that (i) chimeric VSV expressing filovirus GP may not provide an accurate prediction of the neutralizing capacity of antibodies, (ii) EBOV can easily tolerate exchange of GP from other
ebolaviruses or the heterologous marburgvirus or cuevavirus, (iii) swapping the GP genes and expression of a
fluorescent protein from a recombinant filovirus allows for the rapid screening of large number of antibodies
specific for virtually any filovirus, and (iv) sGP serves as a decoy, reducing the effectiveness of virus
neutralization during filovirus human infections.

ACKNOWLEDGEMENTS

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providing the VSV/EBOV-GP virus, Dr. A. Takada (Hokkaido University, Japan) for providing cDNA of LLOV
GP, Dr. T. Geisbert (UTMB) for providing the VSV/BDBV-GP virus, and Drs. Erica Ollmann Saphire and
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GP and the other filoviruses. This project received support from the Defense Threat Reduction Agency (grant
HDTRA1-13-1-0034 to JEC and AB) and U.S. NIH grant U19 AI109711 (to JEC and AB).


FIGURE LEGENDS

Figure 1. Filoviruses are resistant to neutralization by mAbs. Percentage of neutralization of filoviruses EBOV, BDBV or MARV or the corresponding pseudotyped VSVs by human mAbs isolated from survivors. Asterisks indicate concentrations of mAbs which gave different (p < 0.05) percentages of neutralization for filoviruses versus pseudotyped VSVs.

Figure 2. Swapping of GP or their ectodomains with their counterparts from heterologous filoviruses result in viable chimeric filoviruses. A-C. Cloning strategy for generation of EBOV/BDBV-GP, EBOV/SUDV-GP, or EBOV/MARV-GP (A), EBOV/MARV-GPed (B), and EBOV/LLOV-GP (C) full-length clones. D. Schematic representation of the recombinant eGFP-expressing filovirus constructs.

Figure 3. Swapping of filovirus GP has only a minimal effect on the efficiency of viral replication. A. Fluorescent plaques of recombinant wild-type and chimeric filoviruses on day 3 after inoculation of Vero-E6 cell culture monolayers. The micrographs were taken at 10x magnification. B. Growth kinetics of the EBOV chimeras in Vero-E6 cells inoculated at an MOI of 0.1 PFU/cell. The red curve corresponding to EBOV/BDBV-GP is completely hidden under the blue curve representing EBOV/MARV-GP since these two viruses have similar growth kinetics. Asterisks or pound symbol show differences (p < 0.05) in viral titers of the chimeric viruses compared to EBOV on days 1 – 6.

Figure 4. Plaques of chimeric filoviruses can be immunostained by mAbs specific to ectodomains of their GP proteins. A. Vero-E6 cell culture monolayers were inoculated with dilutions of the indicated viruses, covered with 0.9% methylcellulose, and incubated for six days. Viral plaques were immunostained as described in Materials and Methods. B. Top: the degree of difference between the amino acid sequences of BDBV, SUDV or MARV GP versus EBOV GP calculated as 1.00-H where H is position homogeneity (53). Bottom: parts of GP1 and GP2 are designated in colors as follows: SS, signal sequence, RBR, receptor-binding region, GC, glycan cap, MD, mucin-like domain, IFL, internal fusion loop, HR1, heptad repeat 1, HR2,
heptad repeat 2, MPER, membrane-proximal external region, TM, transmembrane domain, CT, cytoplasmic
tail (adapted from reference (54)). Note that the lengths of the proteins, as indicated in the plots, are greater
than that of BDBV, SUDV, EBOV and MARV GP due to the gaps introduced in the alignments.

Figure 5. Chimeric filoviruses are neutralized according to GP ectodomain specificity of antibodies. A.
Binding of filovirus-specific human mAbs to recombinant GP or sGP of the indicated filovirus species. B.
Percentages of neutralization of wild type or chimeric filoviruses by various concentrations of human mAbs.

Figure 6. Use of chimeric filoviruses for high throughput screening (HTS) of mAbs. A. Comparison of the
levels of fluorescence on day 4 after inoculation of cells with 150 or 300 PFU of EBOV/BDBV-GP. B. Levels of
eGFP fluorescence in triplicate Vero-E6 cell suspensions inoculated with 400 PFU of EBOV/BDBV-GP pre-
treated with various concentrations of BDBV41. C. UV fluorescent microscopy of Vero-E6 cells inoculated with
400 PFU of EBOV/BDBV-GP pre-treated with various concentrations of BDBV41; mAb concentrations and the
levels of fluorescence for representative wells I – IV are indicated on Panel B. D. Comparison of the
neutralizing activities of BDBV41 in plaque reduction assay with a biological isolate of BDBV, recombinant
EBOV/BDBV-GP filovirus or pseudotyped VSV (VSV/BDBV-GP), and in HTS with EBOV/BDBV-GP chimera.

Figure 7. The sGP protein reduces virus neutralization by mAb BDBV52. A. Percent neutralization of
EBOV/BDBV-GP or EBOV/BDBV-GPΔsGP by BDBV52 or BDBV41. Disabling of sGP expression makes the
virus partially susceptible to BDBV52. In contrast, both viruses are equally susceptible to BDBV41, suggesting
that the increased susceptibility of EBOV/BDBV-GPΔsGP to BDBV52 is not a result of the altered properties of
viral particles. B. Binding of BDBV52 to both GP and sGP. Parts of GP are designated in colors as in Fig. 4B.
sGP shares with GP the N-terminal part, including SS, RBD, and most of GC, and also has the unique part
(UP) at the C-terminus (13, 14). The most likely location of BDBV52 epitope identified in Flyak et al.,
unpublished, is indicated for both sGP and GP1,2.
**Fig. 1**

Concentration of mAb, µg/ml

% neutralization

10^-1 10^0 10^1 10^2

10^0

50

*

*

*

*

0

50

100

* * *

10^-2 10^-1 10^0 10^1

*

*

*

*

VSV/EBOV-GP

EBOV

VSV/BDBV-GP

BDBV

VSV/MARV-GP

MARV

KZ52

BDBV43

BDBV52

MR201

Concentration of mAb, µg/ml
Fig. 3

A

<table>
<thead>
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<tr>
<td>EBOV/LLOV-GP</td>
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</tr>
</tbody>
</table>

**Limit of detection**

B

Day 0: 1 log10 PFU/ml
Day 1: 2 log10 PFU/ml
Day 2: 3 log10 PFU/ml
Day 3: 4 log10 PFU/ml
Day 4: 5 log10 PFU/ml
Day 5: 6 log10 PFU/ml
Day 6: 7 log10 PFU/ml

**Limit of detection**
### Fig. 4

#### A

<table>
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<tr>
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#### B

![Graph showing viral titers for different strains and GP1/GP2](image)

- **GP1**: SS, RBR, GC, MD, IFL, HR1, HR2, TM
- **GP2**: MPER, CT, SS, RBR, GC, MD, IFL, HR1, HR2, TM
Fig 5

A

KZ52
MR78
BDBV52
BDBV43

BDBV41
MR201
MR246

BDBV43
MR235

B

EBOV

BDBV

MARV-Uga07
EBOV/MARV-GP

KZ52
BDBV41
BDBV43
MR78
MR201
MR235

BDBV52

EBOV
BDBV
SUDV
MARV

EBOV
BDBV
SUDV
MARV

EBOV
BDBV
SUDV
MARV

Concentration of mAb, µg/ml
Neutralization, %
OD$_{405}$ nm

Concentration of mAb, µg/ml
Neutralization, %
OD$_{405}$ nm

Concentration of mAb, µg/ml
Neutralization, %
OD$_{405}$ nm

Concentration of mAb, µg/ml
Neutralization, %
OD$_{405}$ nm

Concentration of mAb, µg/ml
Neutralization, %
OD$_{405}$ nm

GP
sGP
**Fig. 6**

**A**

RLUs, x 10^6

Viral particles, PFU/well

![Graph](image)

**B**

BDBV41 concentrations, log_{10} µg/ml

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</table>

**C**

Neutralization, %

![Images](image)

**D**

Neutralization, %

![Graph](image)

Concentration of BDBV41 mAb, µg/ml
Fig. 7

A

% neutralization

Concentration of mAb, µg/ml

10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2}

EBOV/BDBV-GP

EBOV/BDBV-GPΔsGP

B

sGP

GP1,2

SS RBD GC UP

SS RBD GC MD IFL HR2 TM

GP1 GP2

BDBV41

BDBV52
Cross-Reactive and Potent Neutralizing Antibody Responses in Human Survivors of Natural Ebolavirus Infection

Highlights

- Natural Ebolavirus infection induced B cells encoding cross-reactive antibodies
- Some cross-reactive human antibodies neutralized multiple Ebolavirus species
- A large proportion of BDBV-neutralizing antibodies bound to the glycan cap
- Glycan cap-specific antibodies exhibited very potent neutralizing activity

Authors


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In Brief

Natural Ebola virus infection causes the induction of B cells that encode potent neutralizing human antibodies, which possess, in some cases, a surprising level of cross-reactivity for multiple species of filoviruses. The neutralizing antibody repertoire recognizes diverse features on the surface glycoprotein, but most of the potent antibodies recognize the glycan cap region.
Cross-Reactive and Potent Neutralizing Antibody Responses in Human Survivors of Natural Ebolavirus Infection

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SUMMARY

Recent studies have suggested that antibody-mediated protection against the Ebolaviruses may be achievable, but little is known about whether or not antibodies can confer cross-reactive protection against viruses belonging to diverse Ebolavirus species, such as Ebola virus (EBOV), Sudan virus (SUDV), and Bundibugyo virus (BDBV). We isolated a large panel of human monoclonal antibodies (mAbs) against BDBV glycoprotein (GP) using peripheral blood B cells from survivors of the 2007 BDBV outbreak in Uganda. We determined that a large proportion of mAbs with potent neutralizing activity against BDBV bind to the glycan cap and recognize diverse epitopes within this major antigenic site. We identified several glycan cap-specific mAbs that neutralized multiple ebolaviruses, including SUDV, and a cross-reactive mAb that completely protected guinea pigs from the lethal challenge with heterologous EBOV. Our results provide a roadmap to develop a single antibody-based treatment effective against multiple Ebolavirus infections.

INTRODUCTION

The genus Ebolavirus, family Filoviridae, contains three viral species that are known to cause large deadly disease outbreaks in Africa: Zaire ebolavirus represented by Ebola virus (EBOV), Sudan ebolavirus represented by Sudan virus (SUDV), and Bundibugyo ebolavirus represented by Bundibugyo virus (BDBV). The most recent EBOV outbreak has caused more than 28,000 cases and more than 11,000 deaths (according to the October 14, 2015, World Health Organization [WHO] Ebola Situation Report). While there is no FDA-approved treatment for filovirus infections, several experimental therapeutics against EBOV are being investigated, including small interfering RNAs (Geisbert et al., 2010; Thi et al., 2015), antisense oligonucleotides (Warren et al., 2010, 2015), a nucleoside analog (Warren et al., 2014), therapeutic vaccines (Feldmann et al., 2007; Geisbert et al., 2008), and monoclonal antibody (mAb) cocktails (Olinger et al., 2012; Qiu et al., 2012, 2014). Of these, preliminary treatment studies suggest that the effect of the ZMapp mAb cocktail exceeded the efficacy and treatment window of other experimental therapeutics described so far (Qiu et al., 2014).

The ZMapp cocktail is composed of three EBOV glycoprotein (GP)-specific mAbs (designated c13C6, c2G4, and c4G7) that were isolated initially from mice (Qiu et al., 2011; Wilson et al., 2000), chimerized with human antibody-constant regions, and then produced in Nicotiana benthamiana (Qiu et al., 2014). Single-particle electron microscopy (EM) reconstructions of these mAbs in complex with EBOV surface protein have revealed key sites of vulnerability on the EBOV GP (Murin et al., 2014). One such site lies within the GP base at the GP1/GP2 interface; two of three mAbs from the ZMapp cocktail (c2G4 and c4G7) bind to overlapping epitopes located in this region. The third mAb from the ZMapp cocktail, c13C6, binds a second antigenic site, which is located in the glycan cap region. GP base-region-specific mAbs c2G4 and c4G7 displayed high neutralization activity in vitro (IC50 < 0.1 μg/ml), whereas the glycan cap-specific mAb c13C6 weakly neutralized EBOV only in the presence of complement (IC50 > 1.0 μg/ml) (Qiu et al., 2014). The lower
in vitro neutralization activity of glycan cap-specific antibodies may be due to the removal of the glycan cap by host proteases (Chandran et al., 2005; Côté et al., 2011; Misasi et al., 2012) inside the endosome before GP engagement with the Niemann-Pick C1 receptor (Carette et al., 2011; Côté et al., 2011).

The ability of mAbs to bind to conserved neutralizing epitopes present on the surface of highly variable viral proteins has been documented extensively for HIV (Burton et al., 2012), influenza viruses (Pappas et al., 2014), dengue virus (Rouvas et al., 2015), paramyxoviruses (Corti et al., 2013), and alphaviruses (Fox et al., 2015). Despite similar requirements for virus entry into the cell (Misasi et al., 2012), GPs from BDBV, EBOV, and SUDV strains differ by over 30% at the amino acid level (Towner et al., 2008). This overall genetic divergence among species of genus *Ebolavirus* has hampered the development of ebolavirus cross-neutralizing Abs. The key components of multiple antibody cocktails developed over the last decade neutralize only viruses of species *Zaire ebolavirus*. A weakly neutralizing mAb c13C6 was shown to cross-react with SUDV GPs (Wilson et al., 2000), but it is unknown whether this mAb can neutralize SUDV. Recently, several studies have shown that cross-reactive antibodies in serum can be elicited during natural infection in humans or vaccination of animals. The serum of individuals who survived BDBV, EBOV, or SUDV infections contained ebolavirus cross-reactive IgG, but not IgM (Macneil et al., 2011). Other studies demonstrated that mice immunized with a vaccine bearing the GP of EBOV generated cross-reactive polyclonal mAbs to other ebolaviruses, such as BDBV and SUDV (Meyer et al., 2015; Ou et al., 2012). Four broadly reactive non-neutralizing mAbs were isolated in mice after vaccinating animals with recombinant vesicular stomatitis virus (rVSV) expressing EBOV GP and then boosting initial immune response with the heterologous EBOV vaccine (Meyer et al., 2015). The epitopes recognized by such cross-reactive mAbs are unknown.

In this study, we isolated a large panel of BDBV-specific and ebolavirus cross-reactive mAbs from B cells of survivors of BDBV infection. The results show that a large proportion of mAbs with potent neutralizing activity against BDBV bind to the glycan cap and recognize diverse epitopes within this major antigenic site. We identified several glycan cap-specific mAbs that neutralized multiple *Ebolavirus* species and a cross-reactive mAb that completely protected guinea pigs from the lethal challenge with heterologous EBOV when used as monotherapy. Several of these naturally occurring antibodies exhibit the most potent protective capacity reported, and they possessed unprecedented cross-reactivity for multiple *Ebolavirus* species, including SUDV, for which neutralizing human mAbs have not been reported.

## RESULTS

### Isolation of Human mAbs

To generate human cell lines secreting human mAbs to BDBV, we transformed peripheral blood B cells from seven survivors of the 2007 Uganda BDBV outbreak with Epstein-Barr virus, as described in the Experimental Procedures. To determine the breadth of antibody response in survivors of ebolavirus infection, we screened supernatants from EBV-transformed B cell lines for binding to GPs from diverse representatives of filovirus species: BDBV, EBOV, or Marburg virus (MARV) (Figures 1A and S1). We also used the same GP panel to screen supernatants from transformed B cell lines derived from a survivor of the 2014 EBOV outbreak (Figure 1B) or from a donor who survived MARV infection (Figure 1C). We color coded GP-reactive supernatants based on the cross-reactivity pattern as follows: species-specific cell lines are highlighted in black; and cross-reactive lines to two or three species are shown in yellow or blue, respectively (Figures 1A–1C and S1).

While approximately half of GP-specific B cell lines obtained from BDBV survivors produced antibodies specific to BDBV GP, 24%–50% of GP-reactive B cell culture supernatants also cross-reacted with EBOV GP (Figures 1A and 1D). Similarly, 36% of GP-specific B cell lines obtained from the EBOV survivor cross-reacted with the heterologous BDBV GP (Figures 1B and 1D). Despite the apparent presence of B cells encoding cross-reactive antibodies in survivors of BDBV or EBOV infections to GPs from heterologous *Ebolavirus* species, we detected a very limited cross-reactivity with GPs from MARV, which belongs to a different genus in the family Filoviridae (Figures 1A and 1D). In line with this finding, 90% of GP-reactive B cell lines obtained from the MARV survivor reacted with autologous GP, and only 2% of antigen-specific B cell lines produced *Ebolavirus* cross-reactive Abs (Figures 1C and 1D). The limited cross-reactivity of mAbs to GPs from *Ebolavirus* and *Marburgvirus* species likely is due in part to low sequence conservation between GPs from two genera (only 27% amino acid identity between BDBV and MARV GP) as well as differences in epitope availability caused by different positions of the mucin-like domains on the GP surface of *Ebolavirus* and *Marburgvirus* (Flyak et al., 2015; Fusco et al., 2015; Hashiguchi et al., 2015).

### Binding and Neutralizing Activity of Human mAbs

We fused transformed cells from B cell lines producing BDBV GP-reactive Abs with myeloma cells and generated 90 cloned hybridomas secreting BDBV GP-reactive human mAbs. To determine the breadth of mAb binding, we screened the mAbs...
**Figure 2. Cross-Neutralizing Antibodies from Survivors of Natural BDBV Infection**

(A) Heatmap showing the binding of BDBV mAbs to a panel of filovirus GPs. The EC_{50} value for each GP-mAb combination is shown, with dark red, orange, yellow, or white shading indicating high, intermediate, low, or no detectable binding, respectively. EC_{50} values greater than 10,000 ng/ml are indicated (>). NAb names are highlighted in red.

(B) Heatmap showing the neutralization potency of BDBV GP-specific mAbs against BDBV. The IC_{50} value for each virus-mAb combination is shown. IC_{50} values greater than 10,000 ng/ml are indicated (>). Neutralization assays were performed in triplicate.

(legend continued on next page)
in ELISA-binding assays using recombinant GPs from multiple filoviruses: BDBV, EBOV, SUDV, or MARV GPs. While 33 Abs recognized only the autologous BDBV GP (designated groups 1A and 1B), 20 Abs recognized both BDBV and EBOV GPs (groups 2A and 2B), and 37 Abs recognized all three GPs from BDBV, EBOV, and SUDV (groups 3A and 3B) (Figures 2A and 2C; Data S1). The relative proportions of antibodies that recognize GPs from 1, 2, or 3 Ebolavirus species did not correlate fully with the B cell line frequencies in the initial screen, which can be explained by our prioritization on recovery of a high number of cross-reactive mAbs. We were not able to isolate Abs that bind to the heterologous MARV GP (Figures 2A and 2C; Data S1).

We further characterized the binding of species-specific or cross-reactive mAbs to recombinant GPs by performing a binding assay with the recombinant form of GP that is secreted from the cell to the extracellular space during natural infection (sGP, secreted GP) (Sanchez et al., 1996; Volchkov et al., 1995). While the Ebolavirus GP is a trimer, sGP forms dimers in which each protomer shares only the amino-terminal 295 amino acids with GP. The majority of mAbs recognized epitopes shared between BDBV GP and BDBV sGP (designated groups 1A, 2A, or 3A) (Figures 2A and 2C). We also identified antibodies that bound to BDBV GP, but failed to bind BDBV sGP in ELISA (designated groups 1B, 2B, or 3B) (Figures 2A and 2C). Antibodies from groups 1B, 2B, or 3B also bound the recombinant GP form that lacks highly glycosylated mucin-like domains (BDBV GPΔmuc), suggesting that mAbs from these three groups target epitopes outside of mucin-like domains (Figure S2).

To evaluate the inhibitory activity of isolated mAbs, we tested mAbs in a BDBV neutralization assay. Of the 90 BDBV GP-reactive mAbs, 31 had half-maximal inhibitory concentration (IC50) values <10 μg/ml, and we defined these as neutralizing antibodies (nAbs) (Figures 2B, where nAb names are highlighted in red, and S3). Several nAbs displayed an extremely high neutralizing potency, with IC50 values below 1 ng/ml (Figure 2B). Also, 18 of 31 nAbs bound only to BDBV GP in ELISA, six nAbs recognized BDBV and EBOV GPs, and the remaining seven nAbs bound to GPs from representatives of three Ebolavirus species, BDBV, EBOV, and SUDV. These results suggested that cross-reactive mAbs in our panel might possess neutralizing activity to multiple ebolaviruses. To test this hypothesis, we screened BDBV425 (a group 2A nAb) in an EBOV neutralization assay as the nAb with the lowest half-maximal effective concentration (EC50) value to the heterologous EBOV GP, and we determined that BDBV425 neutralized the heterologous EBOV. Encouraged by this result, we tested nAbs from groups 3A and 3B in EBOV or SUDV neutralization assays to determine whether cross-reactive nAbs can neutralize three Ebolavirus species. We found two cross-reactive nAbs from group 3A (BDBV43 and BDBV324) that neutralized all three ebolaviruses BDBV, EBOV, and SUDV (Figure 2D, BDBV43). The remaining five nAbs from groups 3A and 3B neutralized BDBV and EBOV, but failed to neutralize SUDV (Figure 2D, BDBV289). Analysis of the Ab heavy-chain variable domain sequences for 26 nAbs revealed that all BDBV-specific and cross-reactive nAbs were encoded by unique Ab genes (Table S1).

Major Antigenic Sites Recognized by Human mAbs
To determine whether Abs from distinct binding groups targeted different antigenic regions on the BDBV GP surface, we performed a quantitative competition-binding assay using a real-time biosensor. We tested four BDBV nAbs from binding group 1A, five nAbs from binding group 1B, four nAbs from group 3A, and three nAbs from group 3B in a tandem blocking assay, in which BDBV GP was attached to the biosensor. We also tested five non-neutralizing antibodies from group 1A to determine whether non-neutralizing antibodies target a unique epitope on GP surface. Non-neutralizing and neutralizing mAbs from group 1A and nAbs from group 3A blocked binding of each other to the GP antigen and segregated into a single competition-binding group (Figure 3). These results suggest that mAbs from groups 1A and 3A target a single antigenic region that contains epitopes shared between GP and sGP (Figure 2A). The nAbs from group 3B that did not recognize sGP in ELISA (Figure 2A) segregated into a separate competition-binding group. Group 1B antibodies were interesting in that two nAbs in this group competed for binding with group 3B nAbs, while three nAbs from the group competed for binding with antibodies from group 3A (Figure 3). These findings suggested that there are at least two major antigenic regions recognized by human BDBV nAbs, based on competition-binding studies. The first major antigenic region contains epitopes that both sGP and GP share (recognized by mAbs from groups 1A and 3A) as well as epitopes that are present only on the GP surface (recognized by three mAbs from group 1B). The second major antigenic region contains only epitopes that are present on the GP surface, but not sGP (recognized by two mAbs from group 1B and three mAbs from group 3B).

Diverse Patterns of Molecular Recognition Defined by Negative-Stain EM
To determine the location of the two major antigenic regions targeted by human BDBV nAbs, we performed negative-stain single-particle EM studies using antibodies from groups 1A and 1B. The EM class averages and reconstructions showed clearly that the two major antigenic regions, defined in competition-binding experiments, corresponded to two distinct sites on the GP surface: the glycan cap and the GP base.

Comparison of the structures of glycan cap-directed mAbs from group 1A with those in group 1B revealed that the antibodies have partially overlapping epitopes, but approach the glycan cap at distinct angles (Figures 4A, 4B, and S4). We fitted a previously determined atomic resolution structure of SUDV GPΔmuc (Bale et al., 2012), which reveals more residues of the
glycan cap region than the equivalent EBOV structure, into the envelope of GP from the EM reconstructions, and we determined the regions targeted by each mAb (Figures 4D and 4E). BDBV335, which binds GP and sGP equally well, mainly targets a region between residues 274 and 282. This region appears well defined in the BDBV335 EM map, indicated by the large lobe on the outside of the glycan cap that closely resembles that region in the GP crystal structure. When viewed along the 3-fold axis of GP, BDBV41 binds to the right of BDBV335, further up on the glycan cap, close to a loop that extends from residue 266 to 277. Consistent with this position, we passaged a chimeric VSV in which the G protein was replaced with BDBV GP as a sole surface protein (VSV/BDBV-GP) in the presence of mAb BDBV41 to generate a neutralization escape mutant virus that was completely resistant to the antibody and that possessed two amino acid substitutions, G271R and T272S (Figure S5). The mutation at the 272 position likely explains why BDBV41 is a group 1 antibody, i.e., only recognizes BDBV (with T272), but not EBOV or SUDV (which have the alternate residue K272). BDBV41 also may make contacts with a loop that extends between residues 266 and 277.

![Figure 3](https://example.com/figure3.png)

**Figure 3. BDBV-Neutralizing Antibodies Target at Least Two Distinct Antigenic Regions of the GP Surface**

Data from competition-binding assays using non-neutralizing mAbs from binding group 1A (white background) and neutralizing mAbs from binding groups 1A, 1B, 3A, or 3B (pink background). Numbers indicate the percentage binding of second mAb in the presence of the first mAb compared to binding of un-competed second mAb. The mAbs were considered non-competing if maximum binding of the second mAb was >70% of its un-competed binding (white boxes with red numbers). Numbers indicate the percentage binding of second mAb in the presence of the first mAb compared to binding of un-competed second mAb. The mAbs were considered non-competing if maximum binding of the second mAb was >70% of its un-competed binding (white boxes with red numbers). Gray boxes with black numbers indicate an intermediate phenotype (competition resulted in between 30% and 70% of un-competed binding). Blue, purple, and green dashed lines indicate what appear to be major competition groups; the blue and purple groups overlap substantially, but not completely.
toward the mucin-like domains, from residue 309 to 312 or further in regions that were unresolved in the GP crystal structure. BDBV432 binds to the left of BDBV335, at the top of a helix loop at residues 259–266, and likely makes extensive contacts with a loop from residues around 302–312. Despite a lack of binding to sGP, BDBV432, as well as BDBV353, binds in the glycan cap region, suggesting that these mAbs make contacts with residues that are exclusive to GP.

The other antibodies in group 1B bind to an epitope at the base of GP. These antibodies, including BDBV255 and BDBV259, bind further down on GP than has been observed previously with murine mAbs, possibly contacting residues within GP2 that are part of the membrane proximal external region (MPER) (Figures 4C–4E). These antibodies were refractory to a reconstruction by EM due to predominant side views of the particles and also apparent flexibility. The class averages, however, clearly show that these antibodies bind an epitope that extends down below the base of GP. Three Fabs can be seen in some of the class averages, indicating that despite the apparent small size of this region, three antibodies can be accommodated on one GP trimer. Although the Fabs adopt various positions in each class average, there is not a continuous range of flexibility.

Figure 4. BDBV-Neutralizing Antibodies Bind to the Glycan Cap or Base Region of GP
(A) Shown are negative-stain EM reference-free 2D class averages of group 1A antibodies that bind both the glycan cap of GP and sGP, and group 1B antibodies that bind the glycan cap of GP, but not sGP. BDBV GP or GPmuc was used to generate complexes.
(B) 3D reconstructions of glycan cap binders from groups 1A and 1B reveal that these antibodies bind the glycan cap at overlapping but distinct epitopes. Top (left) and side (right) views of the complexes are shown.
(C) Reference-free 2D class averages of group 1B antibodies (left) reveal that these antibodies bind an epitope below the base of GP that is flexible. In the middle image, GP is colored yellow and each Fab is colored green. The righthand panel illustrates a superimposition of crystal structures of SUDV GPmuc (PDB: 3VE0) and Fabs (PDB: 3CSY) to demonstrate how Fabs may bind to GP.
(D) The composite model delineates the epitopes of the glycan cap mAbs in group 1A or 1B. Side (above) and top (below) views are shown.
(E) Docking a crystal structure of SUDV GPmuc (PDB: 3VE0) (Bale et al., 2012), which contains a more complete model of the glycan cap region targeted by group 1A/B mAbs, reveals how group 1A/B mAbs target a broad region in the GP1 centered on the glycan cap, near the beginning of the mucin-like domains.

See also Figure S4.
since the Fabs themselves are well resolved. These antibodies may require the full MPER and transmembrane (TM) regions, as well as a membrane, in order to bind stably. These features are all lacking in the current recombinant protein used here, a soluble form of the extracellular domain of GP. While the GP2 region is well conserved across the filoviruses, these BDBV-specific mAbs likely bind non-conserved regions in GP2 proximal to the TM region.

**Epitope Mapping of Group 3A mAbs Using Saturation Mutagenesis and Negative-Stain EM**

As the group 3A (cross-reactive) nAbs competed for binding with group 1A (BDBV-specific) nAbs (Figure 3), we hypothesized that some structural features of the glycan cap are conserved among GPs from multiple *Ebolavirus* species. We sought to identify critical amino acids that defined epitopes for three group 3 nAbs (BDBV270, BDBV289, and BDBV324) using a comprehensive EBOV GP alanine-scanning mutation library (Davidson et al., 2015). Epitope mapping identified critical residues for binding by each nAb as follows: W275 for BDBV270, W275 and Y241 for BDBV289, and W275 and L273 for BDBV324. Residues for which mutation reduced binding of three nAbs from group 3A were visualized on the surface of the high-resolution structure of EBOV GP (PDB: 3CSY). This finding suggests that each of these antibodies recognizes overlapping epitopes in the GP glycan cap (Figures 5A and 5B). The previously described murine nAbs 2G4 and 4G7 and the human nAb KZ52 were shown previously to bind the base region of the GP (Lee et al., 2008; Murin et al., 2014), and mutations of the W275 or L273 residue did not reduce the binding of these nAbs (Figure 5C). We passaged VSV/BDBV-GP in the presence of BDBV223 or BDBV289 in an attempt to generate escape mutant viruses, but could not detect...
neutralization-resistant viruses. An isolate passaged in the presence of BDBV223 with a R574H polymorphism in heptad repeat 1 (HR1) region was identified, and for BDBV289 an isolate with an I584M polymorphism in the HR1 region alone or in combination with an E149K substitution in the receptor-binding domain was identified. However, none of these mutations was associated with the ability of those viruses to resist neutralization by the corresponding mAb.

We further characterized BDBV289 by single-particle EM studies of antibody in complex with GP. BDBV289 binds the glycan cap region of GP, centered on the residues W275 and Y241 (Figures 5D and S4). The angle of approach resembles that of the mAb 1H3 from the antibody cocktail ZMab, although 1H3 is specific to EBOV and is weakly neutralizing (Murin et al., 2014; Qiu et al., 2011). Further, BDBV289 also binds sGP, which shares the first 295 amino acids of GP1 with GP, including the glycan cap region (Sanchez et al., 1996; Volchkov et al., 1995). Therefore, despite previous hypotheses that propose that sGP is an immune decoy and that cleavage of the glycan cap prevents neutralizing antibodies from binding this region (Mohan et al., 2012; Murin et al., 2014), we have now identified several antibodies that challenge these ideas. Interestingly, BDBV289 targets an overlapping epitope with antibodies that we identified to be specific to BDBV and that do not bind sGP (Figure 4). Therefore, the glycan cap region is a major antigenic site that contains epitopes with subtle features that influence sGP and GP binding, neutralization, and species cross-reactivity of targeting mAbs.

**Therapeutic Efficacy of Human mAbs in Small Animal Models of EBOV Infection**

To determine the therapeutic activity of cross-neutralizing Abs, we tested several antibodies in mice. We focused on cross-reactive antibodies, and we studied the heterologous effect of BDBV survivor mAbs against EBOV challenge. We selected two nAbs from groups 3A (BDBV289) and 3B (BDBV223) that bound non-overlapping antigenic regions in the competition-binding experiments (Figure 3). The 7-week-old BALB/c mice received 100 μg antibody by the intraperitoneal (i.p.) route 1 or 3 days after inoculation with 1,000 plaque-forming units (PFUs) of mouse-adapted EBOV, strain Mayinga (Bray et al., 1998). BDBV223 and BDBV289 reduced disease and protected mice from death when delivered 1 day after challenge with EBOV (Figure 6). We did not observe a therapeutic effect in the mice receiving the antibodies 3 days after the challenge.

Finally, we set out to test in vivo efficacy of the cross-reactive nAbs BDBV289 and BDBV223 using a guinea pig model of EBOV infection. The 5- to 6-week-old guinea pigs, strain Hartley, were injected with 5 mg antibody by the i.p. route once (day 1) or twice
tion against viruses of the other species. The ability of these highly immunogenic vaccines with proper presentation of GP treatment against multiple ebolaviruses, and they imply that cross-neutralizing mAbs can be used to develop a universal heterologous species of EBOV. These data suggest that showed that they protected animals from lethal challenge with a single treatment against a heterologous EBOV (Figure 7, three of five animals survived) with only a single treatment (Figure 7). We isolated viral RNA from blood of representative animals that were treated with mAbs BDBV223 or BDBV289 but died, and then sequenced the genes encoding the GP (Table S2). Several polymorphisms were detected, but none appeared to be directly related to the epitope specificity of the mAb used for treatment.

DISCUSSION

This study reveals that natural BDBV infection in humans triggers the development of ebolavirus cross-reactive antibodies that target epitopes on the EBOV GP surface confer better protection than treatment with a single mAb alone, we tested the combination of BDBV223 and BDBV289 in guinea pigs. The combination of two antibodies provided complete protection against a heterologous EBOV with only a single treatment (Figure 7). We isolated viral RNA from blood of representative animals that were treated with mAbs BDBV223 or BDBV289 but died, and then sequenced the genes encoding the GP (Table S2). Several polymorphisms were detected, but none appeared to be directly related to the epitope specificity of the mAb used for treatment.

Our study highlights the neutralization and protective potencies of human glycan cap-specific antibodies. It has been suggested previously that glycan cap-binding mAbs may not neutralize well because cathepsins remove this region during viral entry (Murin et al., 2014). However, several of the BDBV glycan cap-specific mAbs isolated here exhibit very potent neutralizing activity, and they recognize diverse epitopes within this major antigenic site. Furthermore, a single glycan cap-specific nAb, BDBV289, provided complete protection in EBOV-challenged guinea pigs. The mechanism used by glycan cap-binding mAbs to neutralize the virus in vitro is unclear. The glycan cap-specific antibodies described here bind to sites distant from the putative cathepsin cleavage site (located at residue 190), so they are unlikely to interfere with GP cleavage. While the amino acid sequence of the GP1 region is generally less well conserved than that of GP2 in viruses of diverse filovirus species, the five neutralizing glycan cap mAbs studied with EM imaging here target conserved residues, indicating that these regions may be important to the viral life cycle. Therefore, these mAbs may block some yet undefined function of the glycan cap.

Several antibody-based treatments provided a complete species-specific protection from EBOV in a non-human primate model of infection (Qiu et al., 2014). However, antibody-based therapeutics against other members of the Ebolavirus genus, such and BDBV and SUDV, are not yet available. While one strategy would be to develop separate antibody treatments for each filovirus infection, an alternative strategy would be to have a universal treatment effective against diverse Ebolavirus species. The development of universal antibody treatments for ebolaviruses seems inevitable, given recent progress in the identification of broad and potent neutralizing antibodies against viruses that exhibit more antigenic diversity than the filoviruses such as HIV (Burton et al., 2012), influenza viruses (Pappas et al., 2014), dengue virus (Rouvinski et al., 2015), alphaviruses (Fox et al., 2015), and paramyxoviruses (Corti et al., 2013). Our results provide a roadmap to develop a single antibody-based treatment effective against multiple Ebolavirus infections. We propose that the principal components of such treatment should include cross-neutralizing mAbs that target conserved elements of the non-overlapping major neutralizing antigenic sites on the GP surface.

EXPERIMENTAL PROCEDURES

Donors
De-identified peripheral blood mononuclear cells (PBMCs) from seven survivors of the 2007 BDBV outbreak in Uganda (Towner et al., 2008) were obtained from a repository at Makerere University (Kampala, Uganda) managed in collaboration with the U.S. Military HIV Research Program MHRP, which is part of the Walter Reed Army Institute of Research. PBMCs were obtained after informed consent from a U.S. survivor of an EBOV infection who was infected while delivering health care in Liberia during the 2014 Ebola virus outbreak with Makona virus. Cells from the EBOV survivor were obtained about 11 weeks after infection and about 7 weeks after discharge from hospital, following several negative PCR tests for presence of virus. PBMCs were obtained from a U.S. survivor of MARV infection who developed the disease in early 2008 following exposure to fruit bats in the Python Cave in Queen Elizabeth National Park, Uganda. This donor’s clinical course was documented previously (Centers for Disease Control and Prevention, 2009), and we have reported previously on isolation of human antibodies from this donor (Fiyak et al., 2015). Peripheral blood from the donor was obtained in 2012, 4 years after the illness, following informed consent. The studies were approved by the Vanderbilt University Institutional Review Board.

Viruses
BDBV strain 200706291 Uganda was isolated originally from the serum of a patient during the first recorded outbreak caused by this virus (Towner et al., 2008) and passaged three times in Vero E6 cells. The virus was provided originally by the Special Pathogens Branch of the U.S. Centers for Disease Control and Prevention (CDC) and deposited at the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA), housed at the University of Texas Medical Branch (UTMB). The chimeric EBOV/BDBV-GP,
Figure 7. Survival and Clinical Signs of EBOV-Inoculated Guinea Pigs Treated with BDBV mAbs
(A and B) Groups of five guinea pigs per group were injected with individual mAbs by the i.p. route 1 day or 1 and 3 days after EBOV challenge, using 5 mg of individual mAb (A) or 5 mg of the combination of two mAbs per treatment (B), as indicated. Animals treated with dengue virus-specific human mAb 2D22 served as controls. The survival curves are based on morning and evening observations. Mortality in the morning is shown in whole day numbers, in the evening in half-day values. The body weight and illness scores are shown with one value per day. See also Table S2.
EBOV/MARV-GP, and EBOV/SUDV-GP constructs expressing EGFP were obtained by replacing the gene encoding EBOV GP with that of BDBV, MARV, or SUDV, respectively (P.A. Ilinikh, unpublished data) and passaged two times in Vero E6 cells. Additional details are reported in the Supplemental Experimental Procedures.

Generation of Human Hybridomas Secreting mAbs

Human hybridomas were generated as described previously (Flyak et al., 2015). In brief, previously cryopreserved samples were transformed with Epstein-Barr virus, CpG, and additional supplements. After 7 days, cells from each well of the 384-well culture plates were expanded into four 96-well culture plates using cell culture medium containing irradiated heterologous human PBMCs (recovered from blood unit leukofiltration filters, Nashville Red Cross) and incubated for an additional 4 days. Plates were screened for BDBV GP antigen-specific antibody-secreting cell lines using ELISAs. Cells from wells with supernatants reacting with antigen in an ELISA were fused with HMMA2.5 myeloma cells using an established electroporation technique (Yu et al., 2008).

Human mAb and Fab Production and Purification

After fusion, hybridoma cell lines were cloned by single-cell fluorescence-activated cell sorting and expanded in post-fusion medium as previously described (Flyak et al., 2015). Hitrap Protein G or Hitrap MabSelectSure columns were used to purify antibodies from filtered supernates. Fab fragments were generated by papain digestion, as described previously (Flyak et al., 2015).

Expression and Purification of Filovirus GPs

BDBV GP ectodomain (BDBV GP, residues 1–637) or the sGP dimer (BDBV sGP, residues 1–516) was used to screen supernatants of transformed B cells. Recombinant GPs were engineered with a C-terminal double strep tag and cloned into a modified M Pertu vector for expression in Drosophila S2 cells. Briefly, plasmids were transfected into S2 cells using Effectene reagent (Qiagen) followed by stable cell selection with 6 μg/ml puromycin. S2 cells first were cultured in Schneider’s medium supplemented with 10% (v/v) fetal calf serum (FCS, Lonza), and later they were adapted to Insect Xpress medium for large-scale expression in 2-l shaker flasks. Stable cells were induced with 0.5 mM CuSO4 and harvested after 4 to 5 days at 27°C. Tangential flow filtration then was used to buffer exchange the supernatants into 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 15 μg/ml avidin (pH 8.0), and target proteins were purified using Strepactin Superflow affinity (Qiagen). GP ectodomains were purified further with S200 size exclusion chromatography (SEC); sGP was purified with S75 SEC. Recombinant ectodomains for EBOV, SUDV, or MARV were designed and expressed similarly.

Screening and EC50 ELISA-Binding Analysis

Soluble forms of the full-length extracellular domain of BDBV, EBOV, SUDV, or MARV GPs or the sGP form of BDBV GP were coated overnight onto 384-well plates at 1 μg/ml. For screening ELISA, 10 μl supernate from a well of a tissue-culture plate was transferred to each well of a 384-well ELISA plate. For EC50 binding analysis by ELISA, purified antibodies were applied to the plates at a concentration range of 30 μg/ml to 170 ng/ml, using 3-fold serial dilutions. The presence of antibodies bound to the GP was determined using goat anti-human IgG alkaline phosphatase conjugate and p-nitrophenol phosphate substrate tablets, with optical density read at 405 nm after 120 min. A non-linear regression analysis was performed on the resulting curves using Prism version 5 (GraphPad) to calculate EC50 values. The Circos software package was used for data visualization (Krzywinski et al., 2009).

EBOV and MARV Neutralization Experiments

Isolated mAbs were screened initially in a high-throughput neutralization assay using EBOV/BDBV-GP with or without 5% guinea pig complement (MP Biomedical) (P.A. Ilinikh, unpublished data). The mAbs that exhibited neutralizing activity also were screened for neutralization of EGFP-expressing EBOV (Towner et al., 2005). Several mAbs were tested for neutralization of EBOV/SUDV-GP and EBOV/MARV-GP by the same approach. Additional information is given in the Supplemental Experimental Procedures.

Biolayer Interferometry Competition-Binding Assay

Competition binding studies using biolayer interferometry and biotinylated BDBV GP (EZ-link Micro NHS-PEG5-Biotinylation Kit, Thermo Scientific 219555) (5 μg/ml) were performed on an Octet RED biosensor (ForteBio), as described previously (Flyak et al., 2015). In brief, the antigen was immobilized onto streptavidin-coated biosensor tips. After a brief washing step, biosensor tips were immersed first into the wells containing first antibody at a concentration of 100 μg/ml and then into the wells containing a second mAb at a concentration of 100 μg/ml. The percentage binding of the second mAb in the presence of the first mAb was determined by comparing the maximal signal of the second mAb applied after the first mAb complex to the maximal signal of the second mAb alone.

Sequence Analysis of Antibody Variable Region Genes

Antibody variable gene sequence analysis was performed as previously described (Flyak et al., 2015). Heavy-chain antibody variable region sequences were analyzed using the IMGT/V-QUEST program (Brouchet et al., 2008; Giudicelli et al., 2011).

EM and Sample Preparation

Fabs were added in 10 M excess to BDBV GpS-Muc and subsequently purified and stained as previously described (Murin et al., 2014).

Image Processing of Protein Complexes

Particles were automatically picked using DoG Picker (Voss et al., 2009) and particle stacks were generated using Appion (Land et al., 2009). Subsequently, reference-free two-dimensional (2D) class averages were generated using iterative multi-reference alignment (MRA)/multivariate statistical analysis (MVA) (van Heel et al., 1996). Non-GP complexes and those with a clear lack of full saturation by Fab were removed to generate a final stack for reconstructions. In some cases, orientation bias or flexibility of Fabs prevented convergence of an acceptable model, although examination of class averages allowed a general assignment of the epitope. Final stack class averages were used to generate initial models using EMAN2 common lines (Tang et al., 2007). A model matching its reference projections was further refined using the entire raw particle stack with EMAN2, as described previously (Murin et al., 2014). For the BDBV41 reconstruction, the EMAN2 reconstruction lacked important features that were present in the class averages, indicating that perhaps some particles lacked full Fab saturation. To circumvent this problem, we utilized the Reion package, which allows three-dimensional (3D) classification to remove particles that may only contain two Fabs, significantly improving the quality of the final EM map (Scheres, 2012). Modeling fitting and EM figures were generated using UCSF Chimera (Pettersen et al., 2004).

Epitope Mapping Using an EBOV GP Alanine-Scan Mutation Library

Comprehensive high-throughput alanine scanning (shotgun mutagenesis) was carried out on an expression construct for EBOV GP (Yambuku-Mayinga variant GP; UniProt: Q05320) (Davidson et al., 2015). Additional details are reported in the Supplemental Experimental Procedures.

In Vivo Testing

The animal protocols for testing of mAbs in mice and guinea pigs were approved by the Institutional Animal Care and Use Committee of the UTMB. The 7-week-old BALB/c mice (Charles River Laboratories) were placed in the ABSL-4 facility of the Galveston National Laboratory. Groups of mice at five animals per group were infected with 1,000 PFU of the mouse-adapted variant GP; UniProt: Q05320 (Davidson et al., 2015). Additional details are reported in the Supplemental Experimental Procedures.
with 1,000 PFU of guinea-pig-adapted EBOV by the i.p. route. Then 24 or 24 and 72 hr later, animals were injected with individual mAbs (5 mg per treatment) or a cocktail of two mAbs (2.5 mg of each mAb per treatment). Animals were weighed and monitored daily for 28 days. After animals became symptomatic, they were examined no less than twice per day. The disease score was calculated using the following parameters: appearance (possible scores 0–3), body condition (0–3), natural behavior (0–3), and provoked behavior (0–3).

**ACCESSION NUMBERS**

The accession numbers for the EM reconstructions reported in this paper are Electron Microscopy Data Bank: EMD-6527 through EMD-6532.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, two tables, and a data file, and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.12.022.

**AUTHOR CONTRIBUTIONS**


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**REFERENCES**


Host-Primed Ebola Virus GP Exposes a Hydrophobic NPC1 Receptor-Binding Pocket, Revealing a Target for Broadly Neutralizing Antibodies

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ABSTRACT The filovirus surface glycoprotein (GP) mediates viral entry into host cells. Following viral internalization into endosomes, GP is cleaved by host cysteine proteases to expose a receptor-binding site (RBS) that is otherwise hidden from immune surveillance. Here, we present the crystal structure of proteolytically cleaved Ebola virus GP to a resolution of 3.3 Å. We use this structure in conjunction with functional analysis of a large panel of pseudotyped viruses bearing mutant GP proteins to map the Ebola virus GP endosomal RBS at molecular resolution. Our studies indicate that binding of GP to its endosomal receptor Niemann-Pick C1 occurs in two distinct stages: the initial electrostatic interactions are followed by specific interactions with a hydrophobic trough that is exposed on the endosomally cleaved GP1 subunit. Finally, we demonstrate that monoclonal antibodies targeting the filovirus RBS neutralize all known filovirus GPs, making this conserved pocket a promising target for the development of panfilovirus therapeutics.

IMPORTANCE Ebola virus uses its glycoprotein (GP) to enter new host cells. During entry, GP must be cleaved by human enzymes in order for receptor binding to occur. Here, we provide the crystal structure of the cleaved form of Ebola virus GP. We demonstrate that cleavage exposes a site at the top of GP and that this site binds the critical domain C of the receptor, termed Niemann-Pick C1 (NPC1). We perform mutagenesis to find parts of the site essential for binding NPC1 and map distinct roles for an upper, charged crest and lower, hydrophobic trough in cleaved GP. We find that this 3-dimensional site is conserved across the filovirus family and that antibody directed against this site is able to bind cleaved GP from every filovirus tested and neutralize viruses bearing those GPs.

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Ebola virus (EBOV) and Marburg virus (MARV) are both members of the Filoviridae family of enveloped negative-strand RNA viruses and are the causative agents of a highly lethal disease for which no approved vaccines or treatments are currently available (1, 2). Due to their virulence and biopotentence, filoviruses are classified as category A pathogens. The ongoing EBOV epidemic in West Africa is the longest and most widespread filovirus outbreak on record (3).

Like all filoviruses, EBOV displays a single virus-encoded protein, the viral glycoprotein (GP), on the surface of the virion. EBOV GP is a 676-residue class I membrane fusion glycoprotein. However, EBOV GP differs from canonical class I fusion proteins, such as those of human immunodeficiency virus and influenza A virus, in that the architecture of its fusion loop more closely resembles those of class II and III glycoproteins (4, 5). EBOV GP is synthesized as a precursor polypeptide, GP0, which assembles into trimers in the endoplasmic reticulum. Each GP0 subunit is then posttranslationally cleaved by the Golgi endoprotease furin to yield disulfide-linked GP1 (≈55 kDa) and GP2 (≈20 kDa) subunits. The final GP assembly, which is an ≈450 kDa trimer of GP1,2 heterodimers, is then displayed on the surface of mature EBOV virions (4, 5). GP1 contains the receptor-binding site and regulates the triggering of the membrane fusion machinery in the GP2 subunit (6). The GP1 structure can be divided into three subdomains: the mucin domain, glycan cap, and GP1 core. The outer mucin domain (GP1 residues 313 to 464), is predicted to be loosely structured and heavily glycosylated, incorporating five N-linked gly-
cans and 12 to 17 predicted O-linked glycans (5). Interior to the mucin-like domain is the glycan cap (GP, residues 227 to 313), which sits atop the GP1 core. The glycan cap is more ordered than the mucin-like domain and contains four N-linked glycosylation sites. Neither the mucin nor the glycan cap domain is essential for viral entry. Indeed, removal of these domains enhances infection by viruses pseudotyped with EBOV GP (7–9). Therefore, it is currently hypothesized that a primary function of the mucin domain and glycan cap is to shield the GP1 core from immune surveillance (4, 5, 10, 11).

EBOV virions are internalized into cells via a macropinocytosis-like mechanism and undergo trafficking to late endosomes (12–15). There, host endosomal cysteine proteases, including cathepsins L (CatL) and B (CatB), cleave GP1 to remove the mucin and glycan cap domains. Cleavage generates a fusion-competent GP trimer (GPCL) comprising the 19-kDa GP1 core domain and GP2 (8, 9, 16). Cleavage of GP1,2 to GPCL is a prerequisite for viral recognition of the host endosomal receptor Niemann-Pick C1 (NPC1) (10, 17–20), strongly suggesting that the receptor-binding site in the GP1 core structure is unmasked by the cleavage of GP1, in late endosomes. Thus, GPCL represents the structure of EBOV GP in a conformation that is competent for receptor binding.

In order to observe possible structural changes in GPCL and to illustrate definitively which surfaces and residues are unveiled upon endosomal proteolysis, we determined the crystal structure of the EBOV GPCL trimer at a resolution of 3.3 Å, in receptor binding.

The crystal structure of EBOV GPCL reveals the NPC1 receptor-binding site in EBOV GPCL which suggested only limited changes in the GPCL structure upon thermolysin digestion (23). The structure of EBOV GPCL is more compact than that of EBOV GP and exhibits more stable crystal packing, less disorder, and improved resolution of X-ray diffraction over that of the previously determined uncleaved GP (4).

New regions of EBOV GP can now be visualized in the EBOV GPCL structure. These include C-terminal residues of GP2, the disulfide link between C53 of GP, and C609 of GP2, and an intra-GP2 disulfide bond between C601 and C610. As EBOV GP2 descends from the base of the GP trimer structure, it forms a tightly ordered loop structure that is stabilized by the intra-GP2 disulfide bond between C601 and C610. This disulfide link turns the peptide chain back toward the body of GP where it is anchored to GP1 by the C53-C609 inter-GP1,2 disulfide bond prior to turning downward toward the transmembrane domain and viral membrane (Fig. 1B).

The most striking structural feature of GPCL is the full exposure of a charged hydrophilic crest and a large hydrophobic trough structure in immediate proximity to the GP2 fusion loop. The trough becomes exposed upon proteolytic excision of the glycan cap from EBOV GP and is 13 Å wide, 23 Å long, and 10 Å deep (Fig. 1C). Residues I113 and L111 form an exposed hydrophobic face inside the trough, while residues V79, T83, W86, F88, L122, V141, and I170 line the bottom of the trough.

Mutation of GP residues exposed after removal of the glycan cap affects viral infectivity and binding to the filovirus receptor NPC1. Previous work utilizing scanning mutagenesis of EBOV GP identified multiple residues important for viral infectivity (24–26). These studies were carried out prior to the availability of a crystal structure of EBOV GP (4) or GPCL (this work) and prior to identification of the endosomal receptor, NPC1 (17, 18). Here, we map these residues onto the crystal structure of EBOV GPCL and determine whether mutations in EBOV GP that reduce infectivity specifically correlate with defects in GPCL-NPC1 binding. Previous work identified three lysines at positions 114, 115, and 140 (16, 25) and hydrophobic residues F88, L111, and L122 (25–27) for which mutation to alanine diminishes infectivity (16, 25). These deficits in infectivity correlate with reductions in NPC1 binding, as determined by co-immunoprecipitation (28).

The crystal structure of EBOV GPCL illustrates that K114, K115, and K140 lie along the crest and F88, L111, and L122 line the trough of EBOV GPCL. These hydrophobic residues are buried in uncleaved EBOV GP (4) but become solvent exposed in the trough of EBOV GPCL. We systematically mutated residues that the GPCL crystal structure shows to be surface-exposed after cleavage, in order to determine their importance for NPC1 binding and viral infectivity and to define the GP1 receptor-binding site (RBS).

We pseudotyped vesicular stomatitis virus (VSV) particles with 73 mutant GP proteins and tested them for viral incorporation of GP relative to the incorporation of the wild-type (WT) protein, and for binding to the conformational antibody KZ52 (4, 21), which only recognizes properly folded GP (see Fig. S4 in the supplemental material). The 68 VSV-GP mutants that met these quality benchmarks were then evaluated for their capacity to recognize a purified, soluble form of human NPC1 domain C in an

RESULTS

The crystal structure of EBOV GPCL reveals the NPC1 receptor-binding site that is unmasked upon endosomal cleavage. Purified EBOV GPCL ectodomains (expressed without mucin-like domains; hereinafter referred to as GP) were treated with thermolysin, which mimics host endosomal protease processing of EBOV GP (8), in order to generate EBOV GPCL trimers for crystallization. EBOV GPCL crystallizes in the space group H3 (R3+H) with four GP monomers and four KZ52 Fab5s in the asymmetric unit (ASU). The ASU contains one full GP trimer and one remaining GP monomer, which itself forms a biologically relevant trimer with two symmetry-related protomers about a crystallographic 3-fold axis. The overall changes to the tertiary structure upon cleavage of GP are minimal, reflected in a root mean square deviation (RMSD) of 0.419 Å compared to the structure of uncleaved EBOV GP (Fig. 1A) (4). This finding corroborates a previous model of EBOV GPCL which suggested only limited changes in the GPCL structure upon thermolysin digestion (23). The structure of EBOV GPCL is more compact than that of EBOV GP and exhibits more stable crystal packing, less disorder, and improved resolution of X-ray diffraction over that of the previously determined uncleaved GP (4).
enzyme-linked immunosorbent assay (ELISA), as described previously (27, 29). We report that WT EBOV GPCL binds to NPC1 domain C with a 50% effective concentration (EC50) of 0.5 nM, consistent with a high-avidity binding interaction between these proteins. In comparison, we find that mutants that demonstrate reduced infectivity are also defective for binding to NPC1 domain C (see Fig. S1 and S2 in the supplemental material). Furthermore, a few single point mutations that cause drastic reductions (>10-fold) in the GPCL-NPC1 domain C-binding EC50 are located in or around the hydrophobic trough and hydrophilic crest. These mutants allow us to map those residues of EBOV GP1 that are critical to NPC1 domain C onto the EBOV GPCL structure and to better
define the RBS (see Fig. S1 and S2). Interestingly, mutation to alanine of two trough residues, F88 and L111, reduces viral infectivity dramatically (by >3 log10 units) but has more modest effects on GPCL-NPC1 binding (see Fig. S2A). The disparity between strong reduction in infectivity but modest effect on NPC1 binding suggests that these residues may be important for steps in viral entry post-NPC1 binding and prior to membrane fusion, such as conformational changes or release of GP2.

The hydrophobic trough exposed on GP1 upon endosomal cleavage is the primary binding site of NPC1 domain C. We performed further mutagenesis of the hydrophobic trough to better define its precise role. Since most of the point mutations to alanine within the hydrophobic trough had only modest effects, we postulated that replacing them with bulkier methionine residues would more completely occlude the trough and prevent GP-NPC1 binding. We selected two trough residues, T83 and I113, which did not inhibit NPC1 binding when mutated to alanine, for additional mutagenesis to methionine (Fig. 2A and B). To prevent misfolding or disruption of the GP structure, we engineered compensatory mutations with interacting residues of the glycan cap to fit the larger methionine residues and prevent steric clashing. We engineered the following mutants: I113M (trough)/F225A (cap), T83M (trough)/F225V+Y232F (cap), and T83M+I113M (trough)/F225A+Y232F (cap) (Fig. 2C to E). For simplicity, since the compensatory mutations are removed along with the glycan cap upon proteolysis, we will only refer to these mutants by the mutations remaining on EBOV GPCL: T83M, I113M, and T83M+I113M. All engineered VSV-GP mutants maintain high levels of incorporation compared to the incorporation of WT GP (see Fig. S2 in the supplemental material). As posited, the single T83M and I113M mutations, as well as the T83M+I113M double mutation, lead to defects in NPC1 domain C binding and pseudovirus infectivity by GPCL bearing them (Fig. 2A to C). We further find that a single point mutation, L122A, located in the bottom of the trough, abrogates both NPC1 domain C binding and pseudotyped virus infectivity (Fig. 2E). The position of L122 suggests that it has a structural role; the L122A mutation may destabilize the local trough structure, preventing NPC1 binding and subsequent infectivity. Together, these findings provide evidence that supports a direct correlation between NPC1 binding and infectivity and effectively maps the GPCL trough as a critical component of the NPC1-binding site.

An overall basic charge on the GP1 crest is required for GP binding to NPC1 domain C and viral infectivity. Experiments performed prior to the identification of the filovirus endosomal receptor NPC1 demonstrated that K114A, K115A, and K140A mutations (now mapped to the GPCL crest) significantly reduce viral infectivity (16, 25). Here, we investigated whether the observed reductions in viral infectivity from these mutations correlate with defects in binding to NPC1 domain C. We show that while the individual mutations K114A and K115A have only modest effects (see Fig. S1 and S2 in the supplemental material), the double mutation (K114A+K115A) dramatically inhibits GPCL-NPC1 domain C binding and viral entry (Fig. 3A and B). In contrast, the K140A mutant showed no significant defect in viral infectivity or NPC1 domain C binding (see Fig. S2). To test the hypothesis that these crest residues participate in electrostatic interactions with NPC1 during virus-receptor engagement, we engineered and analyzed VSV-GPs in which these lysines were replaced with either basic or acidic residues. The K114R+K115R double mutant, which maintains the basic charge, remains fully functional. In contrast, the K114E+K115E double mutant, which reverses charge, displays an even greater deficit in receptor-binding function and entry activity than the neutral K114A+K115A mutant (Fig. 3A and B). To determine whether it is the overall charge of the site or specific basic residues within the site that are important, we mutated two glutamic acid residues in proximity to positions 114 and 115 to alanine. The resulting quadruple mutant (K114A+K115A+E112A+E120A), which is predicted to have WT-like electrostatics, exhibits receptor-binding activity and infectivity at nearly WT levels (Fig. 3A and B). The importance of a set of basic residues but lack of a specific requirement for any one of them individually suggest a need to maintain an overall basic charge on the GPCL crest (Fig. 3C).

Neutralizing antibodies raised from a Marburg virus survivor demonstrate potential panfilovirus neutralization activity. The high degree of sequence and structural conservation in the NPC1-binding site of filovirus glycoproteins makes it an attractive target for the development of broadly neutralizing MAbs with therapeutic potential (see Fig. S3 in the supplemental material). Unfortunately, no such MAbs against ebolaviruses have been isolated. Instead, most known neutralizing anti-ebolavirus MAbs target a conformational epitope at the base of the GP1 trimer (4, 5, 30, 31). Recently, however, several MAbs isolated from a human survivor of MARV infection were found to recognize the hydrophobic GPCL trough and inhibit GP-NPC1 domain C binding (11, 22). Of significance, one anti-MARV MAb from that study, MR72, cross-reacts with purified GP and GPCL of EBOV, while three other MAbs, MR78, MR78, MR111, and MR191, cross-react only with EBOV GPCL (22). MR72, MR78, MR111, and MR191 bind to similar locations on MARV GP but approach from significantly different angles (22). The third complementarity-determining region of the heavy chain variable region (CDRH3) of MR78 binds into the expected MARV GP, RBS (see Fig. S3) (11)

As the RBS is conserved in sequence and structure across known filoviruses, we evaluated the capacity of MR72 and two additional GPCL-reactive antibodies, MR78 and MR191 (22), to recognize and neutralize VSV bearing GPCL from four ebolaviruses (Sudan virus [SUDV], Bundibugyo virus [BDBV], Tai Forest virus [TAFV], and Reston virus [RESTV]) and the cuevavirus Lloviu virus (LLOV) (2, 32). Remarkably, we find that MR72 effectively neutralizes VSVs pseudotyped with GPCL derived from all known filoviruses (Fig. 4A). In contrast, MR191 neutralizes VSV bearing other filovirus GP only weakly, and MR78 fails to neutralize VSVs bearing GPCL derived from any species other than MARV. We speculate that the steeper angle of approach of MR191 to MARV GPCL compared to that of MR78 may enhance the breadth of neutralization by improving access to the shared RBS (Fig. 4A and S3). Of significance, we found that MR72 failed to bind VSVs bearing uncleaved EBOV GP on the surface (see Fig. S3 in the supplemental material). This finding is in contrast to a previous observation of MR72 binding to uncleaved soluble EBOV GP ectodomain (see Fig. S3) (22). It is likely that there are differences in the presentation of EBOV GP on the surface of actual virions that prevent MR72 from binding and effectively neutralizing either wild-type EBOV or VSV bearing uncleaved EBOV GP.

The contrasting neutralization breadth properties of MR72 and MR78, despite their similar binding angles and shared epitope, led us to explore our panel of GPCL mutations to identify
FIGURE 2 Mutagenic occlusion of the EBOV GP1 receptor-binding site. (A) Alanine or methionine mutations were made to key residues in the RBS. The affinities of wild-type and mutant GP1 for NPC1 domain C were analyzed via ELISA. Note that the L122A and T83M+I113M mutations significantly reduce binding to NPC1 domain C. Means ± SD (n = 4) from a representative experiment are shown. (B) Graph displaying titers of VSV pseudoviruses harboring GP1 RBS mutations. Means ± SD (n = 2–4) from a representative experiment are shown. (C) A semitransparent surface has been placed over the cartoon model of the WT RBS on EBOV GP1 to display the RBS pocket (within the dashed oval outline). Residues T83 and I113 are illustrated as sticks (black). (D) Model of EBOV RBS bearing the mutations T83M and I113M (red). The longer side chains of the introduced methionine residues fill the RBS pocket and likely prevent NPC1 domain C binding by occluding the NPC1 binding site. (E) The buried location of L122 (black) is displayed in the EBOV GP1 RBS. See also Fig. S1, S2, and S4 in the supplemental material.
specific residues in the GP RBS that can affect MR78’s neutralization of EBOV GPCL (see Fig. S2 in the supplemental material). We find that a single point mutation, V79A, allows MR78 to neutralize EBOV GPCL: although MR78 cannot neutralize VSV bearing wild-type EBOV GPCL, it can neutralize V79A-bearing VSV-EBOV GPCL (Fig. 4B). Position 79 in EBOV GP is equivalent to position 63 in MARV. Structural alignment of EBOV GPCL with MARV in the MARV GP-MR78 crystal structure (11) suggests that the wild-type V79 may sterically clash with the light chain of MR78. Replacement of valine with the smaller alanine residue (V79A) may improve neutralization by relieving the steric clash (Fig. 4B).

Furthermore, previous studies have shown that, unlike MR72, MR78 fails to block NPC1 domain C binding to EBOV GPCL (11). Therefore, we performed NPC1 domain C competitive-binding assays to determine whether MR78 neutralizes EBOV GPCL-V79A by inhibiting GP-NPC1 binding. Curiously, even though MR78 is now able to neutralize VSV bearing EBOV GPCL-V79A, it remains unable to prevent binding of NPC1 domain C to EBOV GPCL or EBOV GPCL-V79A (Fig. 4B). MR72, however, does block NPC1 binding to EBOV GPCL. Therefore, our data suggest that MAbs MR72 and MR78 may neutralize by distinct mechanisms. MR72 effectively blocks GPCL-NPC1 binding for all filoviruses, whereas MR78 does not block EBOV GPCL-NPC1 binding. We speculate that MR78 neutralizes EBOV entry by inhibiting viral membrane fusion downstream from virus receptor recognition.

In order to gauge the neutralization potentials of MR72 and MR78 relative to those of other MAbs with demonstrated protective efficacy in vivo, we performed a comparative analysis with the combined MAbs of the EBOV-specific ZMapp cocktail: 2G4, 4G7, and 13C6 (31, 33), as well as with KZ52, a known neutralizing MAb from a human survivor (21). Our analysis demonstrates that MR72 can neutralize pseudoviruses at 10-fold lower concentrations of antibody than are required for KZ52 and the ZMapp cocktail (Fig. 4C). Thus, MAbs such as MR72, which target the highly conserved GP1 RBS, represent a novel avenue for both broad and potent neutralization of filoviruses, if they can be delivered to the endosomal compartments where GPCL is generated during entry.

DISCUSSION
In this study, we present the 3.3-Å crystal structure of thermolysin-cleaved EBOV GP (GPCL), which is primed for interaction with the filovirus receptor, NPC1. Thermolysin has previously been demonstrated to mimic host CatB and CatL proteolytic processing of
EBOV GP, which occurs in the endosome and is required for receptor binding and membrane fusion (8–10, 16). This high-resolution structure of EBOV GP has now defined the intermolecular disulfide bridge between C53 in GP1 and C609 in GP2, a region previously unresolved for EBOV GP. The disulfide bridge likely contributes to the inherent stability of ebolavirus GP despite proteolytic processing. This stability is reflected in a high degree of structural conservation between uncleaved EBOV GP (4) and

FIGURE 4 Monoclonal antibodies targeting the conserved GP1 RBS demonstrate panfilovirus neutralization activity. (A) VSV pseudotyped with GPs from different species of filovirus (as indicated in the key to the right) were preprimed with thermolysin to expose the GP1 RBS and then analyzed for reduction in relative infectivity following treatment with MR72 or MR78. (B) The graph to the left shows a comparative analysis of the neutralization of VSV-EBOV GP\textsubscript{C\textsubscript{1}} and VSV-EBOV GP\textsubscript{C\textsubscript{1}}-V79A by MR72 and MR78. The graph to the right displays the results of competitive binding assays detecting NPC1 domain C binding in the presence of increasing concentrations of MR72 or MR78 for EBOV GP\textsubscript{C\textsubscript{1}} and EBOV GP\textsubscript{C\textsubscript{1}}-V79A. The key for both graphs is on the far right. (C) Graph showing the results of comparative infectivity assays of nonprimed VSV pseudotyped with EBOV GP treated with MAbs from the ZMapp cocktail (2G4, 4G7, and 13C6) (33) or the neutralizing EBOV antibody KZ52 (21). MR72 neutralizes primed EBOV GP\textsubscript{C\textsubscript{1}} pseudovirions at \textgreek{nH11022}10-fold lower concentrations than are required for ZMapp or KZ52 to neutralize EBOV GP pseudovirions. See also Fig. S3 in the supplemental material. Means ± SD (\textgreek{nH11006}2–4) from a representative experiment are shown in each panel.
GP<sub>CL</sub>; the aligned structures have an RMSD of 0.419 Å. The crystal structure of EBOV GP<sub>CL</sub> presented here also illustrates how proteolytic priming removes the glycan cap of EBOV GP<sub>P</sub>, to expose the binding site for the filovirus receptor NPC1. The GP<sub>CL</sub> crystal structure suggests that the glycan cap can act as a final layer of defense, shielding the critical and conserved NPC1 domain C binding site from host immune surveillance prior to cellular entry. We show that this RBS has a crest-and-trough morphology and exists at the apex of the GP<sub>CL</sub> trimer.

The crest is lined with hydrophilic basic residues, while the trough is recessed and entirely hydrophobic. Mutagenic analysis of EBOV GP<sub>CL</sub> demonstrates that the crest is involved in nonspecific electrostatic interactions with NPC1, requiring an overall basic charge to facilitate binding of NPC1 domain C. Mutations in EBOV GP (such as K114E, K115E) that reverse the electrostatic charge, not specific amino acid position.

We also further analyzed two mutants with a mutation in the hydrophobic trough, F88A or L111A, which have been described previously as unable to support infection (25, 26). These two mutants are outliers in our analysis. Their infectivities are reduced by more than three log<sub>10</sub> infectious units relative to that of WT EBOV GP, despite only modest defects in binding of NPC1 domain C. We postulate that these NPC1 receptor binds GP in a two-stage process. First, GP<sub>CL</sub> recruits the NPC1 domain C receptor through nonspecific electrostatic interactions with NPC1, requiring an overall basic charge to facilitate binding of NPC1 domain C. Mutations in the crest are initiated between the GP1 RBS trough and NPC1 domain C crest region on GPCL. Without this interaction, there is no detectable GP-receptor binding. Next, specific hydrophobic interactions are initiated between the GP, RBS trough and NPC1 domain C. The specificity of these interactions likely explains the differential effects of individual mutations in the trough (Fig. 3 and 4), whereas the effects of mutations in the crest were determined by charge, not specific amino acid position.

We further analyzed two mutants with a mutation in the hydrophobic trough, F88A or L111A, which have been described previously as unable to support infection (25, 26). These two mutants are outliers in our analysis. Their infectivities are reduced by more than three log<sub>10</sub> infectious units relative to that of WT EBOV GP, despite only modest defects in binding of NPC1 domain C. We postulate that these NPC1 receptor binds GP in a two-stage process. First, GP<sub>CL</sub> recruits the NPC1 domain C receptor through nonspecific electrostatic interactions with NPC1, requiring an overall basic charge to facilitate binding of NPC1 domain C. Mutations in the crest are initiated between the GP1 RBS trough and NPC1 domain C. The specificity of these interactions likely explains the differential effects of individual mutations in the trough (Fig. 3 and 4), whereas the effects of mutations in the crest were determined by charge, not specific amino acid position.

Recent work has identified multiple neutralizing MAbS from a patient who survived MARV infection. The MAbS from those studies were found to bind to the apex of MARV GP<sub>P</sub> (11, 22)—the site we have confirmed here to be the filovirus GP<sub>P</sub> receptor-binding site. Since ebolavirus, marburgvirus, and cuevavirus GP proteins all use the NPC1 protein as a receptor, it is unexpected that the structure of the GP<sub>P</sub> RBS would be highly conserved across all filoviruses (10, 17–19, 32). Thus, we hypothesized that the MAbS identified by Flyak et al. (22), shown to target the RBS trough on MARV GP<sub>P</sub> (11), should be broadly neutralizing. However, unlike the GP proteins of marburgviruses, those of the ebolaviruses and cuevavirus maintain a glycan cap structure that effectively shields the GP<sub>P</sub> RBS from immune surveillance. Therefore, by proteolytically priming filovirus GP<sub>P</sub> on the surface of VSV particles (such as VSV-EBOV GP<sub>CL</sub>), we were able to analyze the neutralization potential of MAbS targeting the otherwise-occluded filovirus RBS. Analysis of GP<sub>CL</sub>-bearing VSVs would tell us if it was worthwhile to target such antibodies to the endosome as future therapeutics.

Of the panel of neutralizing MAbS from an MARV survivor described by Flyak et al. (22), only one, MR72, demonstrates significant cross-reactivity to uncleaved EBOV GP. Three other MAbS, MR78, MR111, and MR191, only react with EBOV GP<sub>CL</sub>. We focused on our analysis on the EBOV GP-reactive MR72 and the EBOV GP<sub>CL</sub>-reactive MR78 and MR191, which approach GP from different angles. Here, we show that MR72 effectively neutralizes VSV pseudovirions bearing GP<sub>CL</sub> from EBOV, SUDV, BDBV, TAFV, RESTV, or LLOV. MR78 neutralizes the EBOV, BDBV, TAFV, and LLOV VSV-GP<sub>CL</sub> virions weakly (and RESTV and SUDV GP<sub>CL</sub> not at all), with infectivity never reduced below 50% (see Fig. S3 in the supplemental material). In contrast, MR78 can only neutralize MARV GP<sub>CL</sub>. It cannot neutralize EBOV GP<sub>CL</sub>-bearing VSVs, even though it is able to bind them.

The crystal structures of MARV GP<sub>CL</sub> and EBOV GP<sub>CL</sub> bound to MR78 suggest that MR78 binds the same site in both viruses. However, the resolution of the EBOV GP<sub>CL</sub>-MR78 complex was too low to identify subtle differences imposed by sequence deviations from MARV GP that might explain why MR78 fails to neutralize EBOV GP<sub>CL</sub> (11). Hence, we used a panel of VSV-EBOV GP RBS mutants to understand which sequence variations could prevent MR78-mediated neutralization of EBOV GP<sub>CL</sub>. Surprisingly, the introduction of a single point mutation (V79A) within the EBOV RBS allows MR78 to neutralize VSV-EBOV GP<sub>CL</sub>-V79A. Of significance, BDBV, TAFV, and RESTV GP's also encode valine at this position, while SUDV and LLOV GP's encode isoleucine and leucine, respectively. The larger Val, Ile, and Leu alphabetic residues encoded by the ebolaviruses and cuevavirus may prevent MR78 from neutralizing their GP<sub>CL</sub>-bearing particles (Fig. 4A; see also Fig. S3 in the supplemental material). We note that, unlike MR78, MR191 exhibits no improvement in neutralization of VSV-EBOV GP<sub>CL</sub>-V79A (see Fig. S3 in the supplemental material). How MR72 but not MR78 is able to overcome divergent amino acids at position 79 to broadly neutralize filovirus GP<sub>CL</sub> is the subject of continued structural and biochemical study.

Remarkably, enhanced neutralization of EBOV GP<sub>CL</sub>-V79A by MR78 was not accompanied by a commensurate increase in its capacity to block NPC1 binding of this GP (Fig. 4). This apparent uncoupling of neutralization and receptor blockade raises the possibility that MR78 may act as an allosteric inhibitor, preventing membrane fusion by binding to GP<sub>P</sub>, subunits in the trimer that are not occupied by NPC1 domain C in the endosome. There, it may inhibit events that occur after receptor binding in order to trigger GP-mediated membrane fusion. Mutations like L111A, which eliminate infectivity without affecting receptor binding, may target these same post-receptor-binding steps.

Recent events, including the unprecedented EBOV epidemic in West Africa (3, 34), coinciding with human cases of MARV emerging in central Africa (35) and the emergence of BDBV (36) and re-emergence of SUDV (37) in this decade, highlight the urgent need for broad-spectrum antifilovirus therapeutics (38). Here, we demonstrate that the highly conserved binding site for the essential intracellular receptor NPC1 provides an attractive and underexplored target for broadly protective antibodies or small-molecule therapeutics. However, one crucial challenge to the development of such antibodies as therapeutics is an evolved feature of the filovirus entry mechanism—the unavailability of its NPC1-binding site to extracellular antibodies. The success of this
antiviral strategy therefore requires novel protein engineering approaches to deliver GPCL-specific MAbs to late endosomes and/or lysosomes, where the NPC1-binding site is unmasked by host proteases.

MATERIALS AND METHODS

Expression and purification of GPCL-KZ52 complex for crystallization. Ebola virus GP (lacking the mucin domain [residues 312 to 462]) was produced by stable expression in Drosophila melanogaster S2 cells. Briefly, Effectene (Qiagen) was used to transfect S2 cells with a modified pMT-puro vector plasmid containing the GP gene of interest, followed by stable selection of transfected cells with 6 µg/ml puromycin. Cells were cultured at 27°C in complete Schneider’s medium for selection and then adapted to Insect Xpress medium (Lonza) for large-scale expression in 2-liter Erlangen flasks. Secreted GP ectodomain expression was induced with 0.5 mM CaSO4 and supernatant harvested after 4 days. Ebola virus GP was engineered with a double Streptag at the C terminus to facilitate purification using Strept-Tactin resin (2-1201-010) (Qiagen) and then further purified by superdex 200 size exclusion chromatography (SEC) in 10 mM Tris-buffered saline (Tris-HCl, pH 7.5, 150 mM NaCl [TBS]). EBOV GPCL was produced by incubation of 1 mg GP with 0.02 mg ther- molysoin overnight at room temperature in TBS containing 1 mM CaCl2 and purified by using Superdex 200 SEC. Trimeric EBOV GPCL was then complexed with a KZ52 Fab fragment prior to crystallization as previously described (4).

Crystallization, data collection, and structure determinations. The purified EBOV GPCL-KZ52 Fab complex was concentrated to 3.3 mg/ml in TBS. The crystal drops consisted of a 1:1 ratio of protein/well solution. The well solution consisted of 25% polyethylene glycol monomethyl ether 550 (PEG MME 550), 10% 2-propanol, 5% ethylene glycol, 100 mM sodium acetate, pH 4.7, and 100 mM calcium chloride. Crystals grew over the course of a month and were flash frozen directly out of the crystal drop into liquid nitrogen for data collection. Data were collected remotely at the Argonne National Laboratory, Advanced Photon Source (APS), from the GM/CA beamline 23-ID-D. The structure was determined using molecular replacement with PHASER (39), within the CCP4 suite (40), using a modified EBOV GP-KZ52 complex model (PDB code 3CSY) with all the residues corresponding to the glycan cap removed (4). Refinement of the EBOV GPCL crystal structure was done through iterative cycles of model building using COOT, followed by refinement with Refmac5 and PHENIX (41–43). Translation/libration/screw (TLS) motion was applied during refinement with the TLS Motion Determination (TLSMD) server used to determine the TLS structure partitions (44, 45). Five percent of the data was set aside prior to refinement for the Rfree calculations for each data set (46). The statistics and stereochemistry of the crystal structure were checked using the MolProbity server until ranking at least at the 95th percentile (see Fig. S2A in the supplemental material) (47). All of the structural figures were rendered using PyMOL (PyMOL Molecular Graphics System, version 1.5.0.4; Schrödinger, LLC).

Cells and viruses. African green monkey kidney (Vero) cells were maintained at 37°C and 5% CO2 in high-glucose Dulbecco’s modified Eagle medium (DMEM; Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal calf serum. Replication-incompetent vesicular stomatitis virus (VSV) (serotype Indiana) pseudotyped viruses were generated as previously described (49). The wild type (VSV-WT GP) encodes enhanced green fluorescence protein (eGFP) in place of the VSV-G gene to allow scoring of infection and bears the EBOV GP muc gene (Mayinga isolate, GenBank accession number AFO86833) but lacks the mucin-like domain (residues 309 to 489 [Δmuc]) (6). Point mutants and multiple mutants were generated by subcloning GP fragments containing the mutation(s) to replace EBOV GP muc. Cleaved VSV-GPCL particles were generated by incubating VSV-GP pseudotypes with thermolysin (250 µg/ml) for 1 h at 37°C. The reaction was stopped by adding phosphoramidon (1 mM) and incubating on ice for 5 min.

Normalization of GP for ELISA. Normalization of GPCL amounts to be used in the binding experiments was done by ELISA, as illustrated in Fig. S4 in the supplemental material. Briefly, high-binding 96-well ELISA plates (Corning) were coated with serial dilutions of GPCL in phosphate-buffered saline (PBS), and allowed to bind at 37°C for 1 h. The plates were blocked with PBS containing 3% bovine serum albumin (PBSA), followed by incubation with the anti-GP monoclonal antibody KZ52 (2 µg/ml in PBS) (21) and a horseradish-conjugated anti-human secondary antibody (Santa Cruz Biotechnology), which was detected by ultra-TMB (3,3’,5’,5’-tetramethybenzidine) substrate (Thermo Scientific). Absorbance readings were subjected to a nonlinear regression analysis (GraphPad Prism software) to generate binding curves and calculate an EC50 value. Additionally, the viroses were normalized for GP incorporation by comparing the amount of GP to the amount of the VSV matrix protein (M). Equal amounts of purified viroses were resolved on SDS-PAGE and blotted for the VSV matrix protein using a mouse anti-VSV M antibody (23H12). Quantification was done using a LI-COR IR dye-conjugated anti-mouse Alexa Fluor 680 secondary antibody (Invitrogen) on the Odyssey Imaging Station and Image Studio 2.1 software (LI-COR Biosciences), and the results were normalized to the WT control. Virus particles that had less than 25% incorporation of mutant GP compared to the incorporation of WT GP or that were highly sensitive to proteolysis were excluded from our analysis.

GP-NPC1 domain C capture ELISA. Binding of GP to NPC1 domain C was performed as previously described (10, 32). Briefly, high-binding 96-well ELISA plates (Corning) were coated with the anti-GP monoclonal antibody KZ52 (2 µg/ml in PBS) (21). Following a blocking step, either uncleaved or in vitro-cleaved GPCL pseudotypes were captured on the plate. Unbound GP was washed off, and serial dilutions of Flag-tagged purified soluble human NPC1 domain C (0 to 40 µg/ml) were added. Bound NPC1 domain C was detected by a horseradish-conjugated anti-Flag antibody (Sigma-Aldrich), using ultra-TMB substrate (Thermo Scientific). EC50s were calculated from binding curves generated by nonlinear regression analysis using GraphPad Prism software. Binding ELISAs were done in duplicate in at least two independent experiments. All incubation steps were done at 37°C for 1 h or at 4°C overnight.

Pseudovirus neutralization assays. Serial dilutions of MAbs and of a no-antibody control were mixed with either cleaved or uncleaved VSV-GP particles and allowed to bind for 1 h at room temperature. Monolayers of Vero cells were inoculated with the antibody-virus mixture in duplicate and incubated at 37°C in 5% CO2. Infection was scored 12 to 16 h postinfection by enumeration of eGFP-positive cells under a fluorescence microscope. The ZMapp cocktail MAbs 2G4, 4G7, and 13C6, as well as MA KZ52, prepared as previously described (33), were generously provided by Mapp Biopharmaceutical. MAbs MR78 and MR72 were prepared as previously described (22).

Protein structure accession number. Coordinates and structure factors have been deposited into the Protein Data Bank under accession number 5HJ3.

SUPPLEMENTAL MATERIAL


Figure S1, PDF file, 2.7 MB.
Figure S2, PDF file, 0.3 MB.
Figure S3, PDF file, 1.2 MB.
Figure S4, PDF file, 0.2 MB.

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REFERENCES


