Developing VHH-based tools to study Ebolavirus Infection

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Submitted to the Microbiology Graduate Program
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

At the

Massachusetts Institute of Technology

June 2019

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ABSTRACT

Variable domains of camelid-derived heavy chain-only antibodies, or VHHs, have emerged as a unique antigen binding moiety that holds promise in its versatility and utilization as a tool to study biological questions.

This thesis focuses on two aspects on developing tools to study infectious disease, specifically Ebolavirus entry. In Chapter 1, I provide an overview about antibodies and how antibodies have transformed the biomedical field and how single domain antibody fragments, or VHHs, have entered this arena. I will also touch upon how VHHs have been used in various fields and certain aspects that remain underexplored. Chapter 2 focuses on the utilization of VHHs to study Ebolavirus entry using VHHs that were isolated from alpacas. Two VHHs were found to neutralize Ebolavirus in both Biosafety Level 2 and 4 laboratory conditions. Ongoing experiments to address mechanism focuses on two aspects of neutralization: Cathepsin inhibition or NPC1-mediated inhibition. Finally, Chapter 3 discusses the overall landscape for Ebolavirus therapeutics and will discuss future directions of this work.

Thesis Supervisor: Hidde L. Ploegh

Title: Senior Investigator, PCMM at Boston Children's Hospital

Acknowledgements

There are many people that I need to thank for supporting me during my graduate studies. I would like to first thank my advisor, Hidde Ploegh, for his guidance throughout my graduate school career. As a leader and professor, he has attracted some of the best people to his lab, creating a fruitful environment for research. Your wisdom and insight have led to many unique projects that challenged my scientific curiosity.

I would also like to thank my committee, Michael Laub and Lee Gehrke for being supportive over the many years. Our conversations have helped me both scientifically as well as keeping me grounded. Thank you both for providing assistance during our lab's move to Boston Children's Hospital. I would also like to thank Sean Whelan for reading my thesis and agreeing to be on my committee as an examiner.

Next, I would also like to thank all members of the Ploegh lab from the Whitehead Institute and Boston Children's Hospital for being great colleagues and friends through the years. I would like to specially thank Dr. Thibault Harmand, Dr. Nick McCaul, Dr. Matthias Truttmann, and Lotte Wijne for their friendship and willingness to take the much-needed coffee breaks to discuss politics, TV shows, and the occasional science. Nick: for being a great friend and colleague. Your willingness to always help and teach people really speaks volumes. But also, to introducing me into Dungeons and Dragons. Thibault: Your energy and craziness meant that there has never been a dull moment in the lab. It has always been great working with you both in the lab and out. Matthias: for your wisdom and leadership and your willingness to dispense advice. Lotte: thank you for being a good friend. Whether it be bonding over food, music, corgis, or open houses. I'll miss our time in lab. I would also like to thank Joy Xie and Nova Pishesha for being great bay mates at Whitehead: we had so much fun traveling, eating, and bonding over whatever food Boston sent our way. Djenet Bousbaine, whose bike chats I really value, but also for being a great friend and colleague. For reading my thesis and providing feedback, I would like to thank Djenet Bousbaine, Ross Cheloha, and Nick McCaul for their valuable comments.

I would like to thank past Ploegh members. I would like to the Leo Hanke and Florian Schmidt for really introducing me into the lab and helping me get started on my thesis work. Jasdave Chahal: your infectious energy really invigorated the Whitehead crew. Our continued conversations outside the lab were very valuable and helpful in navigating graduate school.

I would also like to thank my Microbiology Program cohort: Josh, Nate, and Joseph. Your friendship and support over the years has been incredibly helpful especially when our lab moved to Harvard Medical School.

Special thanks to our lab manager, Robert Miller. Thank you for keeping the lab afloat and taking care of equipment whenever the occasion arises. Thank you to Simona Stella for dealing with the day-to-day administration of our lab.

I would finally like to thank my family for always being supportive during the at-times difficult road of graduate school. I know how hard it was for them to see me leave California, but their visits to Boston were always happy reminders of the West Coast.

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Chapter 1

Introduction

An Introduction to Ebolavirus

Ebolavirus (EBOV) is the causative agent of Ebola virus disease, a viral hemorrhagic fever resulting from EBOV infection. EBOV was first discovered in 1976 in the Democratic Republic of the Congo (formerly known as Zaire) and South Sudan. Between 1976 and 2014, there have been over twenty documented epidemics caused by three species of EBOV: *Zaire ebolavirus, Sudan ebolavirus, and Bundibugyo ebolavirus* (Malvy, McElroy, de Clerck, Gunther, & van Griensven, 2019). These outbreaks accounted for approximately 2400 cases and 1600 deaths (**Table 1**). In 2014, the largest Ebolavirus epidemic began, spanning multiple countries in Western Africa. By its conclusion in 2016, there were 28,652 cases and 11,325 deaths attributed to *Zaire ebolavirus* infection (Kaner & Schaack, 2016). As of spring 2019, the second largest Ebolavirus outbreak is ongoing in the Democratic Republic of the Congo. Despite extensive international intervention, the 2014-2016 outbreak highlighted the need for a better understanding of Ebolavirus infection and considerations for therapeutics.

Ebolavirus Genome

Ebolaviruses belong to the genus *Ebolavirus* of the family *Filoviridae* in the order *Mononegavirales*. This order consists of viruses that have a single-stranded RNA genome of negative polarity. Within this genus, there are six known species, four of which cause disease in humans: *Zaire ebolavirus, Taï Forest ebolavirus, Bundibugyo ebolavirus, and Sudan ebolavirus.* The remaining two cause disease in nonhuman primates and pigs (*Reston ebolavirus*) and bats (*Bombali ebolavirus*).

The approximately 19 kilobase genome of Ebolaviruses encodes seven genes: nucleoprotein (NP), viral protein (VP) 30, polymerase cofactor (VP35), matrix (VP40), VP24, polymerase (L) and glycoprotein (GP) (**Figure 1**). Each of the seven genes fulfill a specific role in facilitating successful EBOV infection. Each gene is sequentially transcribed into mRNA by the polymerase, L, an RNA-dependent RNA polymerase responsible for genome replication and transcription of viral genes. NP encapsulates the genome, forming the viral ribonucleoprotein complex. VP30 is a phosphoprotein responsible for transcription initiation (Xu et al., 2017). VP35, an essential cofactor for L, also plays a role in interferon antagonism (Prins, Cardenas, & Basler, 2009). VP40, the major matrix protein, facilitates viral budding at the host membrane surface (Bornholdt et al., 2013). The function of VP24 is not well understood but it is thought to play a role in immune evasion and virion formation (Banadyga et al., 2017). GP encodes the glycoprotein that mediates entry into host cells. GP is the sole polycistronic gene in the EBOV genome. GP mRNA is edited by the viral polymerase complex, producing three glycoprotein isoforms with different functions: transmembrane GP, soluble GP (sGP), or second small GP (ssGP) (Mehedi et al., 2011). Expression of viral genes facilitates the production of new viruses and assists in mounting a successful infection.

Ebolavirus Infection

Ebolavirus is transmitted through contact with contaminated bodily fluids via open wounds or mucus membranes. EBOV infects multiple cell types in humans and non-human primates and results in a poor antibody response in non-survivors (Baize et al., 1999).

Initial targets of EBOV are antigen-presenting cells, such as dendritic cells or macrophages. Infected cells travel to draining lymph nodes where viral replication continues, thereby allowing further dissemination (Geisbert et al., 2003; Baseler, Chertow, Johnson, Feldmann, & Morens, 2017). Shortly thereafter, infection spreads to the liver and spleen (Malvy et al., 2019). Infection of endothelial cells causes cellular necrosis, leading to vascular permeabilization, compromising the structural integrity of the endothelium (Wahl-Jensen et al., 2005). Eventually, tissue damage occurs in response to virus-associated cytopathic effects and massive release of inflammatory cytokines (Zarschler, Witecy, Kapplusch, Foerster, & Stephan, 2013).

Viruses have evolved sophisticated strategies to avoid recognition by the immune system. Likewise, EBOV encodes specific viral proteins to suppress the immune response. Immune suppression is mediated primarily by two viral proteins expressed during replication: VP35 and VP24. VP35 was identified as an antagonist of type I interferon response by way of binding to double-stranded RNA, leading to suppression of RIG-I mediated signaling (Cardenas et al., 2006). This, in turn, blocks IRF-3-dependent induction of interferon α and interferon β. Similarly, VP24 inhibits interferon production by prevent translocation of STAT1 into the nucleus, effectively blunting STAT1-dependent interferon production (A. P. Zhang, Abelson, et al., 2012; A. P. Zhang, Bornholdt, et al., 2012). It is unclear whether inhibition of STAT1 translocation is a consequence of direct VP24-STAT1 interaction or through binding of karyopherin $\alpha 1$, a nuclear import protein (Reid et al., 2006). By means of this two-pronged approach, which perturbs the signaling pathways responsible for interferon production, Ebolavirus effectively evades the immune response.

Ebolavirus Glycoprotein

Successful infection of EBOV depends on GP, an approximately 450 kDa trimeric glycoprotein complex responsible for attachment, fusion, and entry into host cells. Prior to assembly of trimeric GP, the GP precursor (GP_0) is cleaved by a furin-type protease in the Golgi into GP_1 and GP_2 subunits, which remain linked by a disulfide bond (**Figure 2**). While many receptors have been implicated in EBOV attachment to the plasma membrane, none have been shown to be solely necessary for entry thus far. Upon interaction with DC-SIGN/L-SIGN (Simmons et al., 2003), LSECtin, hMGL (Takada et al., 2004), β-integrins (Takada et al., 2000), or Tyro3 receptors (Shimojima et al., 2006), virions are internalized by macropinocytosis and trafficked to late endosomes. Proteolytic cleavage by cathepsins B and L activates the glycoprotein, priming it for fusion. It does so by removal of the mucin-like domain (MLD), exposing the receptor binding domain (RBD) in GP_1. The newly revealed RBD then interacts with Niemann Pick C1 (NPC1), its entry receptor, by binding to the loop C domain of NPC1 (NPC1-C) (**Figure 3, Figure 4**). Proteolytic cleavage also potentiates GP_2, liberating the internal fusion loop (IFL), which projects into the host membrane (Cote et al., 2011; Brecher et al., 2012; Spence, Krause, Mittler, Jangra, & Chandran, 2016). Following GP-mediated membrane fusion between the host and viral membranes, the viral genome is released into the cytoplasm where sequential transcription of viral genes by polymerase, L, begins.

Ebolavirus GP is a class I fusion protein. This class of fusion proteins, which includes the prototypical Influenza A virus (IAV) hemagglutinin (HA), are distinguished by proteolytic priming of a single protein precursor into its functional constituents (Harrison, 2015). GP differs in many ways from HA. HA_0 is cleaved into HA_1 and HA_2, containing a functional receptor binding site

and the fusion loop respectively, while GP_0 cleavage into GP_1 and GP_2 by furin-like proteases does not yield the same functional constituents (**Figure 2**). Instead, $GP_{1,2}$ requires further cleavage by endosomal cathepsins after internalization to render GP fusion-competent. While structural studies of GP-mediated fusion have not yet elucidated the specific conformational states of GP required for successful fusion, it occurs in NPC1-positive late endosomes through interaction with the NPC1-C loop C domain (Miller et al., 2012; Bornholdt et al., 2016; Spence et al., 2016; H. Wang et al., 2016) (**Figure 4**).

Aside from its central role in host entry, GP also influences viral replication and pathogenicity. The large structure of GP is believed to displace adhesion proteins needed for cellular attachment, as seen by cell rounding of infected and transfected cells (Takada et al., 2000). This is presumably responsible for the increased permeability of the endothelial barrier (Z. Y. Yang et al., 2000; Wolf, Beimforde, Falzarano, Feldmann, & Schnittler, 2011). Additionally, mRNA editing of GP during transcription results in a soluble, dimeric GP that lacks a transmembrane domain (sGP) (**Figure 1**) (Lee & Saphire, 2009). sGP is found in the circulation of infected individuals (Maruyama et al., 1999) and acts as an "antibody sink," thus blunting the humoral immune response (Pallesen et al., 2016). ssGP, another soluble form of GP, is expressed by EBOV-infected Vero E6 cells, but no function has been yet identified (**Figure 1**) (Mehedi et al., 2011). Therefore, GP consists of multiple isoforms that subvert the immune response and potentially exacerbate infection.

Modes of virus neutralization

Viral neutralization can be achieved through neutralizing antibodies that block the function of critical viral proteins. Neutralizing antibodies curtail infection by interfering with receptor interactions or by inhibiting fusion between the host and viral membranes (**Figure 5**).

Interference of virus-receptor interactions

Neutralizing antibodies can block attachment of virus to host cells by preventing engagement of a viral protein to its cognate receptor. For example, some neutralizing antibodies inhibit human immunodeficiency virus (HIV-1) infection by blocking or occluding the CD4-binding site of gp120, one of the receptors required for HIV-1 entry (Dalgleish et al., 1984; Kwong et al., 1998; Saphire et al., 2001; Raja, Venturi, Kwong, & Sodroski, 2003).

Inhibiting host-virus membrane fusion

Antibodies that bind the virus may not prevent receptor engagement but instead prevent fusion between viral and host membranes. Overcoming the large kinetic barrier presented by the fusion of two lipid bilayer membranes requires large conformational changes of viral fusion proteins (reviewed in (Harrison, 2005, 2008)). By restricting these conformational changes, antibodies that target conserved epitopes involved in the fusion transition should block infection and prevent release of the viral genome into the host cytoplasm (Ekiert et al., 2009). Palivizumab, an antibody against respiratory syncytial virus (RSV) F protein, is an example of blocking cell-to-cell fusion through restriction of the conformational changes required for fusion (Huang, Incognito, Cheng, Ulbrandt, & Wu, 2010; Swanson et al., 2011).

Vaccine-based virus protection

Vaccines can elicit adaptive immune responses via activation of B and T lymphocytes. Protection against viral pathogens can be achieved through B and T cell activation. Activated B cells can secrete antibodies that recognize the vaccine immunogen. $CD4^+$ T cells assist in class switching and affinity maturation of these B cells. Finally, $CD8^+$ T cells can directly kill infected cells.

Vaccines fall into many categories: live, attenuated, subunit-based, inactivated, toxoid, conjugate, DNA, and recombinant vector vaccines. Each of these vaccine strategies carries its own advantages and disadvantages.

Most vaccines that confer protection are believed to induce the production of antigen-specific neutralizing antibodies. There is now increasing evidence that some vaccines induce antigen-specific T cells to provide long-lasting protection. In the field of HIV-1, T-cell based vaccines are attractive based on evidence that $CD8^+$ T cells predominate in controlling and eradicating HIV-1 infection (Borrow et al., 1997). DNA vaccines are used to induce $CD8^+$ T cell responses. Through administration of an DNA plasmid encoding the antigen of interest, induction of $CD8^+$ T cells is observed. For example, following intramuscular injection of a DNA vaccine encoding *Plasmodium falciparum* PfCSP, antigen-specific $CD8^+$ T cells were elicited that targeted infected hepatocytes in both mice and humans, providing protection against infection (Sedegah, Hedstrom, Hobart, & Hoffman, 1994; R. Wang et al., 1998). Likewise, DNA vaccination a plasmid encoding the IAV nucleoprotein also induced MHC-I restricted $CD8^+$ T cells and protected immunized mice against a lethal challenge of IAV (Fernando et al., 2016).

Antibody-dependent enhancement of infection

Although neutralizing antibodies provide protection from viruses, some antibodies may also paradoxically promote infection. In the case of dengue virus (DENV) infection, there are multiple serotypes: DENV-1, DENV-2, DENV-3, and DENV- 4. Serotype-specific neutralizing antibodies of DENV are unable to neutralize different serotypes. Instead, these enhancing antibodies bind to the envelope protein and are internalized along with the virus in an FcR-dependent fashion (Goncalvez, Engle, St Claire, Purcell, & Lai, 2007). Hepatitis C infection can be enhanced in a similar manner, in which neutralizing antibodies at a sub-neutralizing concentration do not neutralize but instead enhance infection by internalization via FcRI, FcRII, or FcRIII (Meyer, Ait-Goughoulte, Keck, Foung, & Ray, 2008).

Ebolavirus Therapeutics

The 2014-2016 Ebolavirus epidemic highlighted the need for novel Ebolavirus therapeutics. Due to the rapid expansion of the outbreak, clinical trials were fast-tracked in order to provide therapeutic options to combat this public health threat. Many candidate therapeutics have emerged since, and these fall in two categories: small molecule inhibitors or immunotherapeutics (G. Liu et al., 2017).

Vaccine Development against Ebolavirus

Vaccination plays an important role in providing protection to individuals during outbreaks. For Ebolavirus, there are currently limited options for vaccinations strategies. However, as the development of promising candidates was accelerated during the 2014-2016 epidemic, several are now undergoing trials (**Table 2**).

Recombinant vesicular stomatitis Indiana virus, pseudotyped with Ebola-GP (rVSV-EBOV), has emerged as a promising vaccine for use against Ebolavirus. rVSV-EBOV was reported to have efficacy in Phase III trials in Guinea, with no cases reported after vaccination (Henao-Restrepo et al., 2017). Vaccinations of at-risk populations were performed during the 2018 Ebolavirus outbreak to protect individuals who might have been in contact with infected individuals (Malvy et al., 2019). Other VSV or adenovirus-based vector vaccines (**Table 2**) are undergoing preclinical trials.

Small Molecule Inhibitors for Ebolavirus

Small-molecule inhibitors have primarily targeted the RNA-dependent RNA polymerase, L. One class of these inhibitors are nucleoside analogs that block RNA polymerase function and prevent further synthesis of viral genes. BCX4430, or Galidesivir, is an adenosine nucleoside analog that prevents RNA-polymerase termination once it is converted into its active triphosphate form (Warren et al., 2014; Taylor et al., 2016). Incorporation of BCX4430 during RNA-dependent RNA synthesis leads to termination of RNA synthesis. The Phase I trial for BCX4430 has been completed, but the results have not yet been made public. At this time, it is unclear whether there will be a Phase II trial for this drug.

Anti-sense RNA molecules are also under consideration for their potential as a therapeutic against EBOV. TKM-Ebola (Arbutus Biopharma), a lipid nanoparticle containing three small-interfering RNAs (siRNAs) targeting VP24, VP35, and L, protected rhesus monkeys against infections with Ebolavirus (Thi et al., 2015). However, drug development has been suspended following development of flu-like symptoms during initial trials.

Phosphorodiamidate morpholino oligomers (PMOs) are synthetic antisense molecules that can bind mRNAs and block their translation. AVI-7537, a PMO specific to VP24, was administered to Rhesus monkeys infected with Ebolavirus. Six of the eight animals in the study survived with no detectable viral RNA in the sera 8 days post-infection (Warren et al., 2015). Despite promising Phase I outcomes, development of this drug has been halted by Sarepta Therapeutics due to funding constraints and has yet to be revisited.

Ebolavirus Immune therapeutics

The use of antibodies as post-exposure prophylaxis has historically been successful for treatment of several infectious diseases, such as rabies, RSV, cytomegalovirus, and vaccinia (Keller & Stiehm, 2000). As GP is the only surface-exposed protein on EBOV, it is the preferred target of immune therapeutics. Only recently have antibodies shown promising outcomes in prophylactic treatment in non-human primate models, highlighting the challenging nature of developing therapeutic antibodies for EBOV infection (Group et al., 2016).

While various monoclonal antibodies against EBOV have been generated and tested in animal models, many of them fail to protect in non-human primate models (Gonzalez-Gonzalez et al., 2017). For example, KZ52, one of the best characterized monoclonal antibodies against GP, failed to protect against a lethal challenge in non-human primates, even at a high antibody concentration of 50 mg/kg despite efficiently inhibiting viral fusion in cell culture (Oswald et al., 2007; Davidson et al., 2015) (**Figure 6**). ZMapp is one of the few passive immunization strategies with demonstrated use for post-exposure prophylaxis of EBOV infection. ZMapp is composed of a cocktail of three antibodies: 13c6, c2G4, and m4G7. Despite its successful

protection, its scalability limited deployment during the 2014 epidemic. Expanding the range of available antibody therapeutics would clearly be beneficial.

While vaccines against Ebolavirus are beginning to demonstrate promising field results, therapeutics for post-exposure prophylaxis have not seen the same level of progress. Heavy-chain only antibodies provide a complementary antibody-based approach for studying Ebolavirus infection and neutralization.

Single Domain Antibody Fragments

Camelid-derived single domain antibody fragments, or the variable domain of the heavy chain of a heavy chain only antibody (VHH), can provide a complementary approach to the use of conventional antibodies, which consist of two identical heavy and light chains.

Heavy chain only antibodies are expressed by all Old World (Bactrian and dromedary camels) and New World (llamas, guanacos, alpacas, and vicuñas) camelids (**Figure 7**). Heavy-chain only antibodies do not associate with light chain and lack the C_H1 domain which normally pairs with the light chain constant region (Hamers-Casterman et al., 1993; Ingram, Schmidt, & Ploegh, 2018)(**Figure 8**). VHHs do not require interaction with light chain variable regions due to the mutations from hydrophobic to more hydrophilic residues at positions 37, 44, 45, and 47 (**Figure 8**). This removes the hydrophobic interface necessary for variable heavy and light chain interaction (Achour et al., 2008). The variable regions of heavy chain only antibodies therefore do not rely on pairing of the heavy and light chain. Thus, a heavy-chain only antibody can be truncated to its minimal unit, a 15 kDa VHH or nanobody (**Figure 7**). VHHs retain the same binding affinity as the full-length antibody from which they are derived. The minimal unit of

conventional antibodies that retains antigen binding requires expression of the variable heavy (V_H) and variable light (V_L) chains connected by a small linker, known as a single chain variable fragment (scFv) (Ahmad et al., 2012). Unlike VHHs, scFvs are prone to aggregation and require more extensive optimization to be practical (Gil & Schrum, 2013).

Compared with conventional antibodies, VHHs have many advantages that make them attractive for biotechnological use. The antigen binding fragment is smaller in VHHs than in scFvs, as they do not rely on the interaction with a variable light chain. Their small size improves tissue penetration (Cortez-Retamozo et al., 2004), a property desirable for drug delivery in cancer treatments and other applications where tissue penetration is important. VHHs can be easily expressed in *Escherichia coli* at high yields of up to 200 mg/L ((Zarschler et al., 2013), as they do not require disulfide bonds or N-glycans for either folding or antigen binding. Furthermore, the longer CDR3s in VHHs have led to their use in identifying epitopes found in enzyme active sites (De Genst et al., 2006) as well as conserved epitopes buried in trypanosome proteins, which are typically inaccessible by conventional antibodies (Lauwereys et al., 1998; Stijlemans et al., 2004). VHHs may be a valuable tool to probe the function of EBOV-GP. Only few VHHs against this antigen have been reported (Liu, Shriver-Lake, Anderson, Zabetakis, & Goldman, 2017).

VHHs as Antivirals

Given the widespread use of conventional antibodies as antivirals, it should be no surprise that VHHs are also beginning to be explored in the context of treating infectious

diseases. Indeed, the ability of VHHs to recognize cryptic antigens coupled with their ease of production makes them an attractive alternative to conventional immune therapeutics.

Antiviral VHHs are of great interest because of their propensity to recognize cryptic epitopes in viral antigens that are inaccessible to conventional immunoglobulins. VHHs, such as ALX-0171, a VHH against RSV, show promising therapeutic potential (Detalle et al., 2016). VHHs have been described that block infection of many different pathogens: IAV, RSV, Rabies virus, poliovirus, foot-and-mouth disease virus, rotavirus, HIV-1, hepatitis B virus, porcine retrovirus, vaccinia virus, Marburg virus, tulip virus X, and bacteriophage p2 have all been targeted by VHHs (Vanlandschoot et al., 2011). Other VHHs target intracellular viral antigens such as the IAV and VSV nucleoprotein have been discovered and show antiviral effects (Ashour et al., 2015; Hanke et al., 2016; F. I. Schmidt, L. Hanke, et al., 2016; Hanke et al., 2017). Although not useful from a clinical perspective, such VHHs can provide insight into molecular aspects of virus-host interactions. The growing list of antiviral VHHs illustrates a growing interest in antiviral VHHs not only as therapeutics, but also as important tools to study different aspects of viral infection.

VHHs as Tools for Biological Research

VHHs are not only limited to their use as therapeutics or industrial purposes. Many groups have explored their use as tools to study biological processes.

VHHs have been used extensively to characterize diverse cellular processes. Intracellular expression of inhibitory VHHs helped characterize stages of IAV and VSV viral lifecycle (Hanke et al., 2016; F. I. Schmidt, L. Hanke, et al., 2016; Hanke et al., 2017) Enzymatic activity can be modulated by VHHs. For example, Huntingtin-associated yeast interacting protein E (HypE)

AMPylation activity was modulated using activating or inhibitory VHHs (Truttmann et al., 2015; Truttmann et al., 2016). Stabilization of an intermediate of inflammasome assembly was confirmed by the use of a VHH specific for a component of the inflammasome. (F. I. Schmidt, A. Lu, et al., 2016). Due to the small size of VHHs, microtubule organization has been also studied through the use of tubulin-specific VHHs in super-resolution microscopy, allowing for visualization of individual microtubules (Mikhaylova et al., 2015). VHHs have great potential as tools in assisting biologists to better understand proteins of interests through the perturbation of normal function or stabilization of intermediate states.

VHHs are of interest to crystallographers as well, because VHHs can be used as chaperones to aid in crystal formation. G-protein coupled receptors (GPCRs) are notoriously difficult to crystalize, especially when studying specific conformational states. The structure of the β_2- adrenergic receptor was crystallized by using a VHH chaperone as an agonist to stabilize the active state (S. G. Rasmussen et al., 2011). Many other structures for GPCRs and type IX secretion systems have been resolved by using this approach to elucidate the crystal structure for a variety of proteins (Duhoo et al., 2017; Che et al., 2018). Thus, VHH interaction with enzymes can help stabilize particular conformational states and affect enzymatic activity.

VHHs provide a versatile toolkit in which to probe biological function by means of perturbation or stabilization of a protein of interest. My thesis focuses on developing tools to study infectious diseases through the use of VHHs and heavy chain only antibodies. To do this, I will use VHHs to study aspects of Ebolavirus infection.

Figures

Country	Cases	Deaths	Species	Year
South Sudan	284	151	*Sudan ebolavirus*	1976
Dem. Rep. of Congo	318	280	*Zaire ebolavirus*	1976
Dem. Rep. of Congo	1	1	*Zaire ebolavirus*	1977
South Sudan	34	22	*Sudan ebolavirus*	1979
Côte d'Ivoire (Ivory Coast)	1	0	*Taï Forest ebolavirus*	1994
Gabon	52	31	*Zaire ebolavirus*	1994
Dem. Rep. of Congo	315	250	*Zaire ebolavirus*	1995
South Africa	2	1	*Zaire ebolavirus*	1996
Gabon	60	45	*Zaire ebolavirus*	1996
Gabon	37	21	*Zaire ebolavirus*	1996
Uganda	425	224	*Sudan ebolavirus*	2000
Republic of Congo	57	43	*Zaire ebolavirus*	2001
Gabon	65	53	*Zaire ebolavirus*	2001
Republic of Congo	143	128	*Zaire ebolavirus*	2002
Republic of Congo	35	29	*Zaire ebolavirus*	2003
South Sudan	17	7	*Sudan ebolavirus*	2004
Uganda	149	37	*Bundibugyo ebolavirus*	2007
Dem. Rep. of Congo	264	187	*Zaire ebolavirus*	2007
Dem. Rep. of Congo	32	15	*Zaire ebolavirus*	2008
Uganda	1	1	*Sudan ebolavirus*	2011
Uganda	6	3	*Sudan ebolavirus*	2012
Dem. Rep. of Congo	36	13	*Bundibugyo ebolavirus*	2012
Uganda	11	4	*Sudan ebolavirus*	2012
Dem. Rep. of Congo	66	49	*Zaire ebolavirus*	2014
Multiple countries	28652	11325	*Zaire ebolavirus*	2014-2016
Dem. Rep. of Congo	8	4	*Zaire ebolavirus*	2017
Dem. Rep. of Congo	54	33	*Zaire ebolavirus*	2018
Dem. Rep. of Congo	ongoing	ongoing	*Zaire ebolavirus*	2018

Table 1: List of Ebolavirus Outbreaks (1976 - present)

Adapted from "Ebola Virus Disease Distribution Map: Cases of Ebola Virus Disease in Africa Since 1976." By Center for Disease Control and Prevention. Retrieved May 14, 2019 from https://www.cdc.gov/vhf/ebola/history/distribution-map.html.

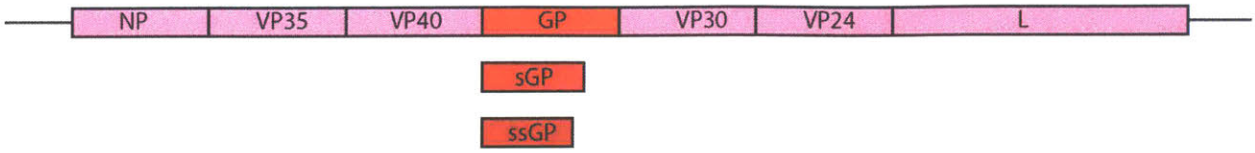

Figure 1: Ebolavirus Genome

Ebolavirus is a negative-stranded, non-segmented RNA virus. Its genome encodes 7 genes: nucleoprotein (NP), viral protein (VP) 30, polymerase cofactor (VP35), matrix (VP40), VP24, polymerase (L) and glycoprotein (GP). Through mRNA editing, GP can be translated into 3 isoforms: GP, soluble GP (sGP), or second soluble GP (ssGP). Each GP isoform contributes to successful virus infection.

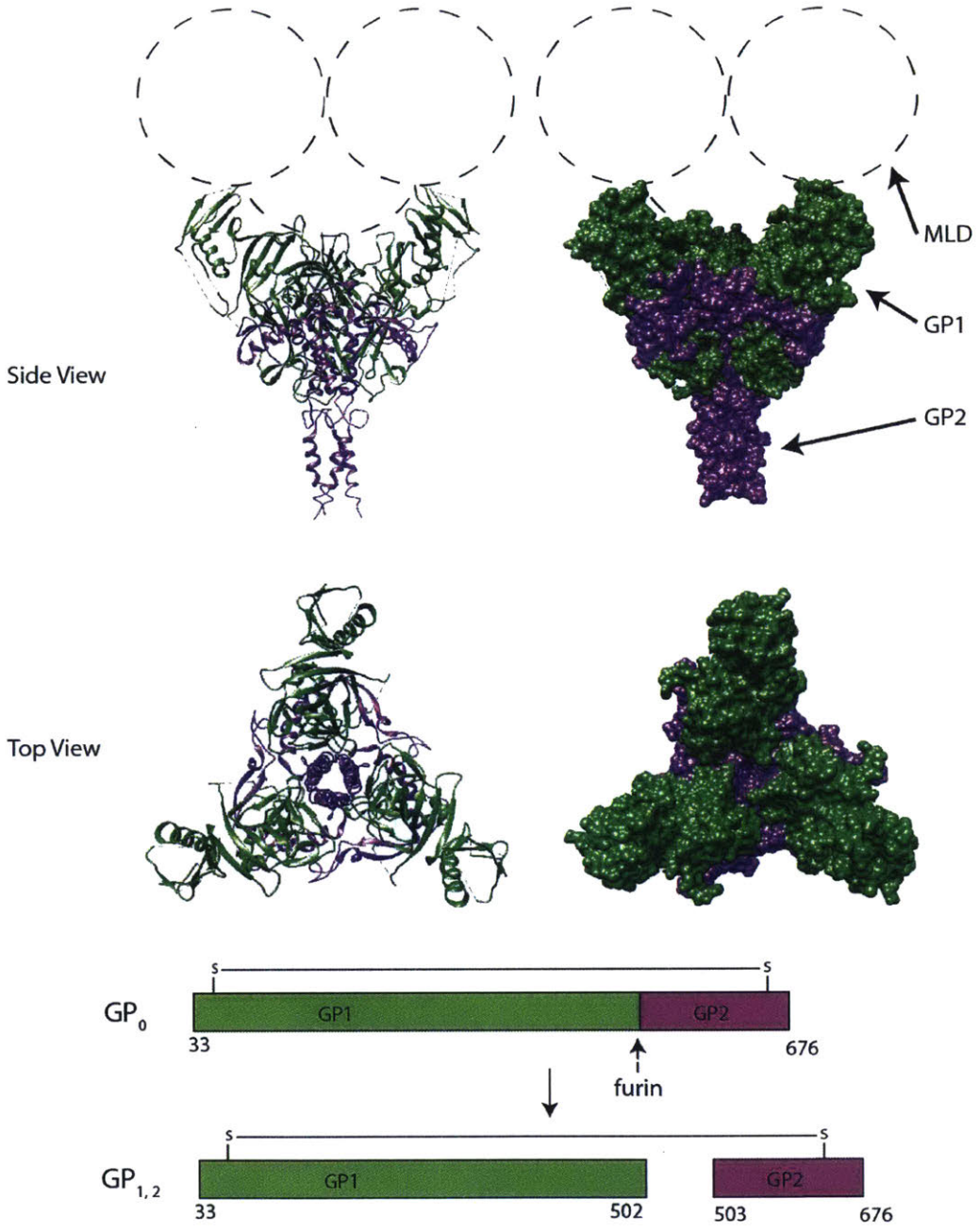

Figure 2: Crystal Structure of Ebolavirus Glycoprotein

Ebolavirus GP is expressed as a precursor, GP_0. Cleavage by furin in the Golgi results in disulfide-linked GP_1 (green) and GP_2 (purple). The large mucin-like domain (MLD) is a highly glycosylated portion of GP_1 with 8 N-linked and 80 predicted O-linked glycosylation sites.

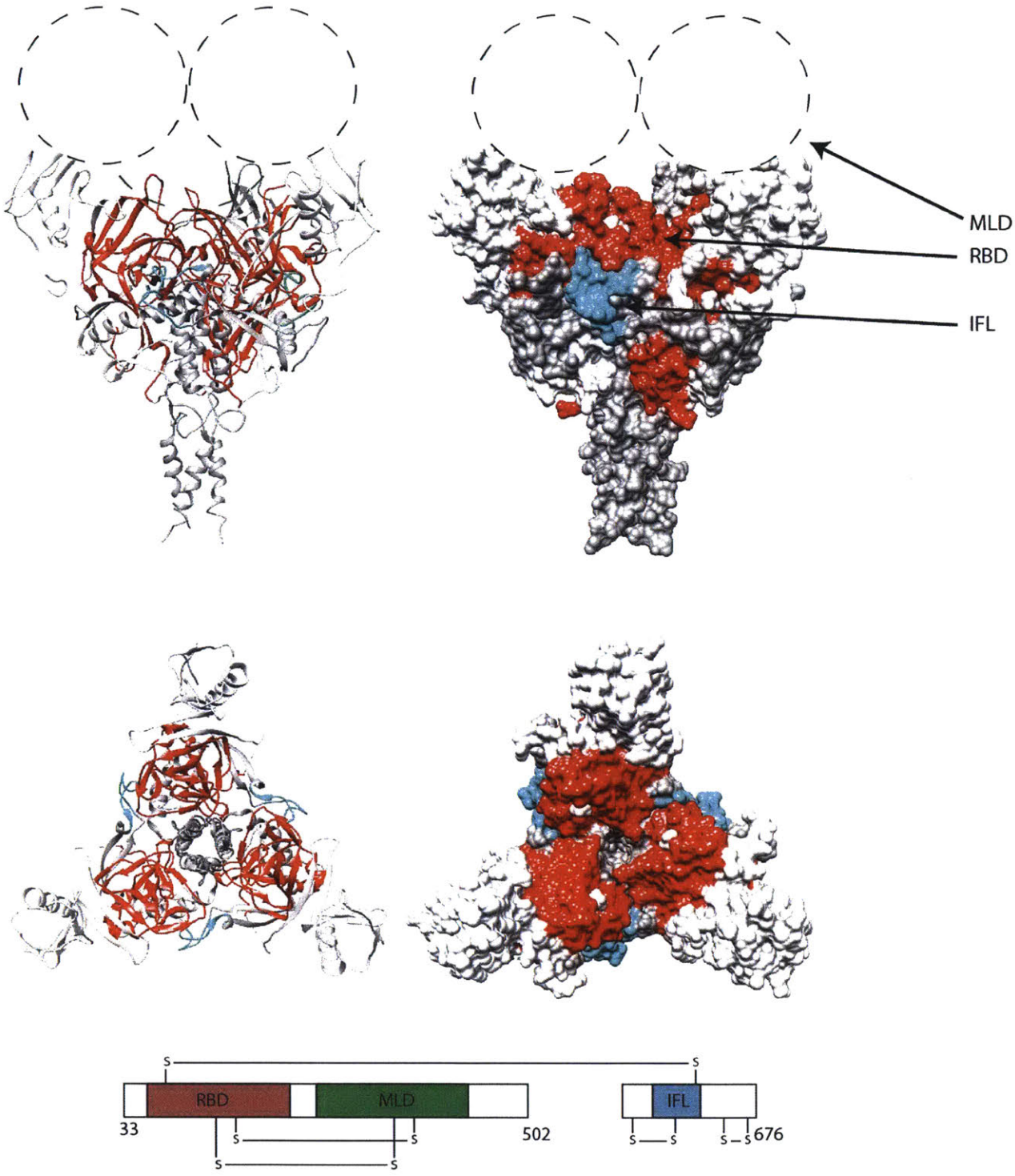

Figure 3: Functional domains of GP are hidden by the mucin-like domain (MLD)

Prior to infection, the functional domains of EBOV are hidden by the MLD in its full-length form. The receptor binding domain (RBD) in GP_1 is uncovered only upon cleavage by endosomal proteases, cathepsins B and L, removing the mucin-like domain (MLD). The internal fusion loop (IFL) in GP_2 is also liberated, allowing for eventual fusion with the host membrane.

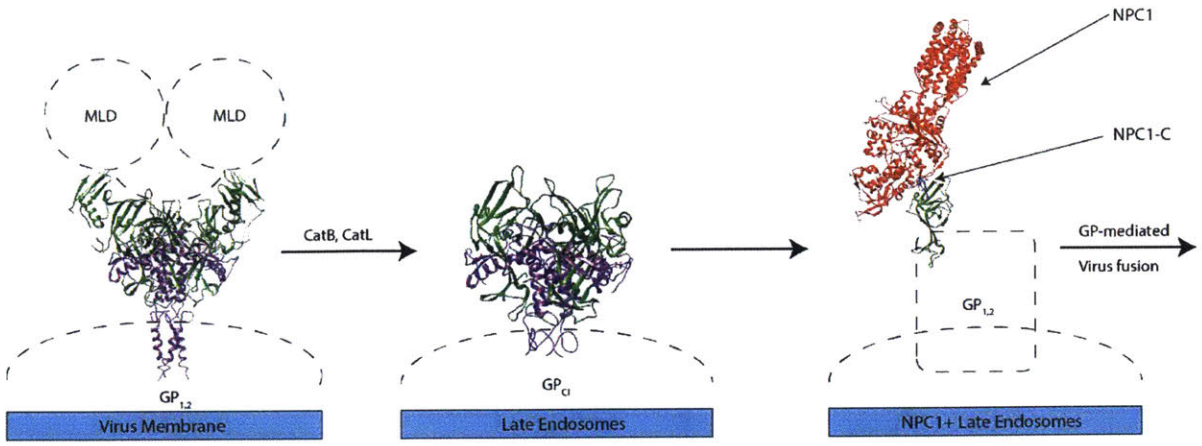

Figure 4: EBOV GP processing in host cells

On the EBOV membrane, GP is expressed as $GP_{1,2}$ containing a mucin-like domain (MLD). Upon internalization and trafficking to late endosomes, GP is proteolytically cleaved by host cathepsins B and L, which removes the MLD. This results in cleaved GP (GP_{Cl}). Upon proteolysis, the receptor binding domain is revealed, allowing binding to NPC1 (via NPC1-C). This interaction precedes fusion of the viral and host membranes. (PDB: 5JNX, 3CSY, 5JQ3)

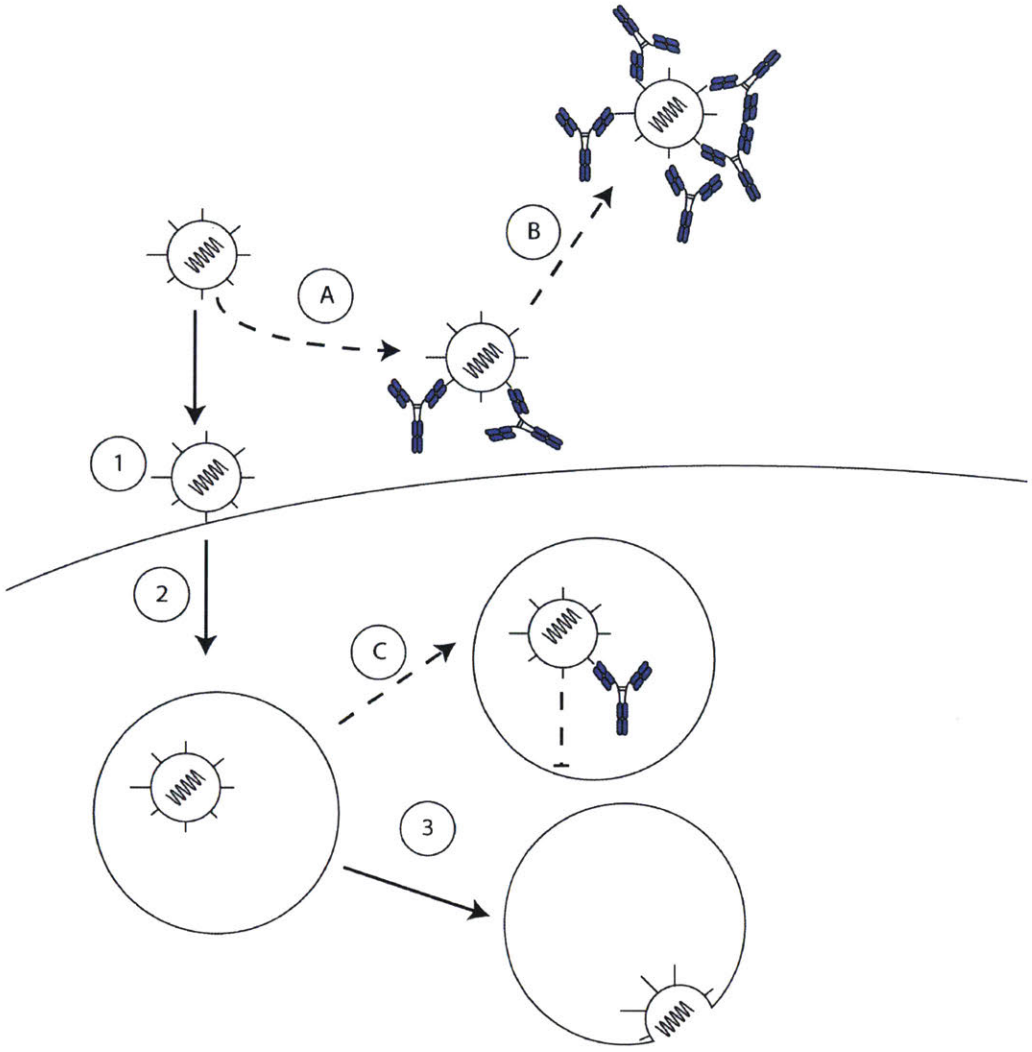

Figure 5: Modes of antibody-mediated virus neutralization

In the course of infection of a cell, enveloped viruses first attach to host receptors via viral entry proteins (1). Viruses are internalized (2) and trafficked through endosomal compartments where they may interact with host factors. Eventually, the virions will fuse with the host membrane and release the infectious genome (3).

Antibodies might interact with viral proteins to prevent infection (A) by interfering host receptor engagement (B) or preventing fusion altogether (C).

	Leading company or institution and country of origin	Vector	Administration
Recombinant VSV-ZEBOV	Merck (USA)	VSV	Single dose
ChAd3-EBO-Z with or without MVA-BN-Filo	GlaxoSmithKline (UK) and, for MVA-BN-Filo, Bavarian Nordic (Denmark)	Chimpanzee adenoviral serotype 3 or MVA	Single dose or heterologous prime-boost regimen
Ad26.ZEBOV with MVA-BN-Filo	Johnson & Johnson (USA), and MVA-BN-Filo from Bavarian Nordic (Denmark)	Human adenoviral serotype 26 or MVA	Heterologous prime-boost regimen
Ad5-ZEBOV	Academy of Military Medical Sciences and CanSino Biologics (China)	Human adenoviral serotype 5	Single dose or homologous prime-boost regimen
GamEvac-Combi	Gamalei Scientific Research Institute of Epidemiology and Microbiology (Russia)	VSV and Ad5-vectored vaccine	Heterologous prime-boost regimen

Table 2: Summary of Promising Vaccines during the 2013-2016 Epidemic.

Adapted from "Ebola Virus Disease" Prof Denis Malvy, MD; Anita K McElroy, PhD; Hilde de Clerck, MD; Prof Stephan Günther, MD; Prof Johan van Griensven, MD. By The Lancet. Retrieved May 14, 2019 from https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(18)33132-5/fulltext#seccestitle110.

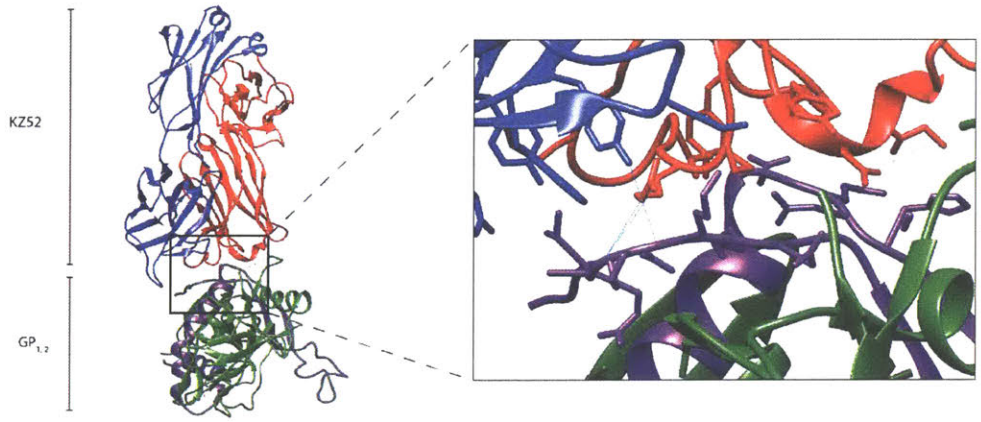

Figure 6: Interaction of EBOV GP with neutralizing human antibody KZ52 blocks fusion

The human monoclonal antibody KZ52 neutralizes EBOV by preventing fusion. KZ52 (red: KZ52 heavy chain, blue: KZ52 light chain) interacts with GP_2 and is thought to prevent transition from the pre-fusion to post-fusion conformations (Oswald et al., 2007; Lee et al., 2008; Davidson et al., 2015). KZ52 binds to the internal fusion loop of GP_2 (purple) and interacts with the interface of GP_1 (green) and GP_2 (purple). PDB: 5HJ3

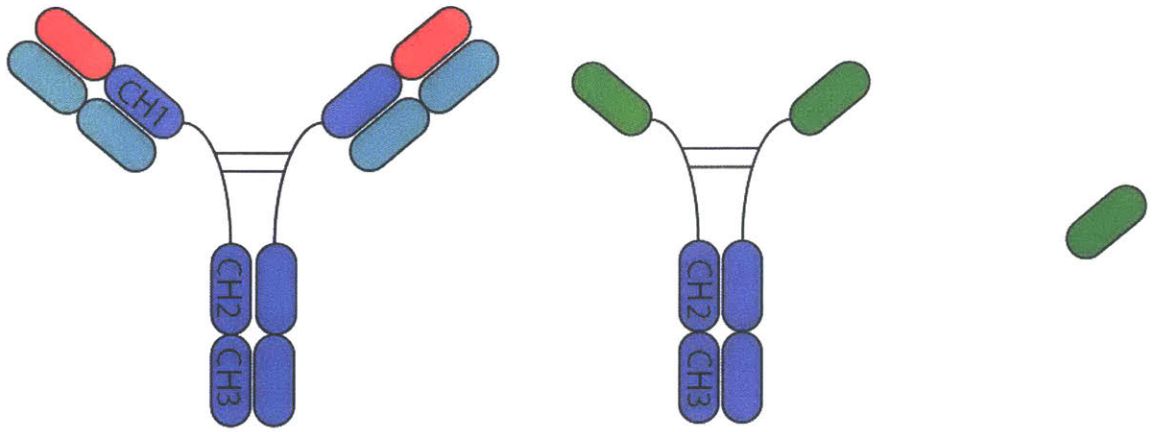

Figure 7: Comparison of Conventional Antibodies and VHHs

Camelids express both conventional antibodies and heavy chain-only antibodies. Heavy chain only antibodies lack a CH1 domain, resulting in a molecular mass of 100 kDa. Due to the lack of cognate light chain interaction, the variable region of heavy-chain only antibodies can be isolated as a 15 kDa VHH.

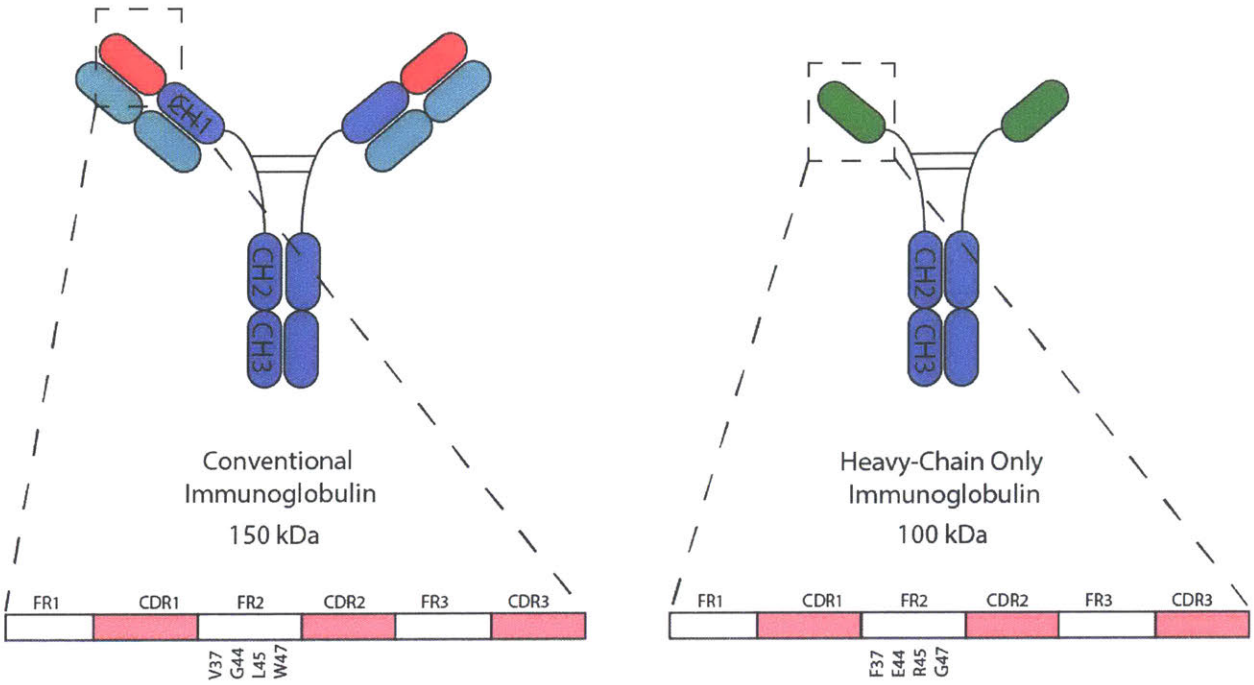

Figure 8: Hydrophilic substitutions remove the need for cognate light chain interaction

The germline encoded V-region in heavy-chain only antibodies contains non-synonymous mutations in FR2 at positions 37, 44, 45, and 47. A change from hydrophobic to hydrophilic residues removes the hydrophobic interface of variable heavy chain with the variable light chain.

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Chapter 2

Neutralizing Single Domain Antibody Fragments against Ebolavirus

(Manuscript in progress)

Abstract: Camelid-derived single domain antibody fragments (VHHs) have found application in different therapeutic and research settings. Here, we report the use of VHHs to neutralize Zaire Ebolavirus. We identified two VHHs that recognize the Ebolavirus glycoprotein (GP). These VHHs neutralize Ebolavirus and exhibit antiviral activity similar to conventional antibodies that neutralize infection. We determined that these two VHHs, G10 and G84, block infection of Ebolavirus by two different mechanisms. G10 blocks attachment of the virus, whereas G84 blocks infection once viral attachment has occurred.

Introduction

Ebolavirus is a negative-stranded, non-segmented RNA virus that belongs to the family of *Filoviridae*, viruses known to cause viral hemorrhagic fever. Discovered in 1976, Ebolavirus spreads rapidly between individuals through blood contact or contaminated bodily fluids. This rapid transmission complicates the design of effective containment and treatment strategies (Matua, Van der Wal, & Locsin, 2015). The 2013-2016 epidemic underscored the need for therapeutics and vaccines to contain such explosive expansion of Ebolavirus infection. To date, there are no vaccines proven to be efficacious in field studies, although there are several vaccine candidates that are currently undergoing clinical trials.

Ebolavirus (EBOV) infection critically depends on a Type I transmembrane protein, glycoprotein (GP), a ~ 450 kDa trimeric complex responsible for the attachment, fusion and entry of the virion. Prior to assembly, GP precursor (GP_0) is cleaved by a furin-type protease and gives rise to GP_1 and GP_2 linked by a disulfide bond (Chandran, Sullivan, Felbor, Whelan, & Cunningham, 2005). Following attachment to the surface of cells, virions are internalized and trafficked into the late endosomes where they encounter cathepsins B and L. Proteolytic cleavage by these enzymes activates the glycoprotein to achieve its fusogenic form (Chandran et al., 2005; Schornberg et al., 2006; Kaletsky, Simmons, & Bates, 2007; Hood et al., 2010; Brecher et al., 2012; Spence et al., 2016). It does so by removing the mucin-like domain, thus revealing its receptor binding site in GP_1, required for binding to Niemann Pick C1, its entry receptor (Carette et al., 2011; Bornholdt et al., 2016; Spence et al., 2016; H. Wang et al., 2016; M. K. Wang, Lim, Lee, & Cunningham, 2017). In addition, GP_2, which contains the fusion loops and heptad repeats, is liberated to project into the host membrane when NPC1 engagement

occurs. The requirement of these steps for infection immediately suggests targets for intervention, but does not obviate the need for alternatives to the currently available options. (Johansen et al., 2013; Hong et al., 2014; Choi, Hong, Hong, & Lee, 2015; Bornholdt et al., 2016; van der Linden et al., 2016; Schafer et al., 2017; Flyak et al., 2018; Fuentes, Ravichandran, & Khurana, 2018; Gunn et al., 2018; Saphire, Schendel, Gunn, Milligan, & Alter, 2018; S. Yang et al., 2018).

Antibodies as agents for prophylaxis or post-exposure treatment are attractive opportunities to curtail viral infection. As a therapeutic strategy, currently the best option is to target GP (Lee & Saphire, 2009). The large mucin domain located C-terminally of GP_1, a 150-residue stretch consisting of 8 N-linked and 80 predicted O-linked glycosylation sites, forms an "umbrella" that can interfere with antibody recognition. Indeed, this large mucin domain is thought to prevent antibodies from recognizing GP (B. Wang et al., 2017). The mucin-like domain is removed once internalization has occurred and has been cleaved via cathepsin B and L proteolysis (Lee et al., 2008). Only a few antibodies have been identified that can block infection. For example, KZ52, an antibody that binds to the base of GP and interferes with fusion, is the reference antibody commonly used for neutralization studies; however it fails to protect macaques upon EBOV challenge (Oswald et al., 2007). Further complicating our understanding of GP inhibition, there are few high-resolution crystallographic information available; thus, limiting our understanding of the molecular determinants of Ebolavirus neutralization and protection (Saphire, Schendel, Fusco, et al., 2018). Expanding the breadth of antibodies available for functional and structural experiments is imperative to better understand and otherwise elusive antigen target.

The variable domains of camelid-derived heavy chain-only antibodies (VHHs or nanobodies) can serve as alternatives to conventional, full-sized antibodies in many applications. Their small size improves tissue penetration and enables the construction of multivalent derivatives that recognize distinct epitopes on one and the same antigen, as shown for VHHs that neutralize botulinum toxin. VHHs can be produced in high yield in *E. coli* and show excellent thermostability. The complementarity-determining-region (CDR) CDR3 of VHHs is typically longer than that seen in conventional antibodies. These longer CDRs can protrude into protein crevices at the target protein's surface. This trait is exemplified by a VHH that recognizes HIV GP120: its ability to protrude into the CD4 binding pocket on GP120 is held responsible for its uniquely broad neutralizing ability across the different clades of HIV (McCoy et al., 2012). The epitopes recognized by VHHs can thus be quite distinct from those recognized by conventional antibodies (S. G. Rasmussen et al., 2011), offering the opportunity of complementary strategies to achieve neutralization of EBOV.

Antibodies can neutralize viruses by different mechanisms. They may directly interfere with adsorption of the virus to surface receptors or prevent conformational transitions required for infectivity. For example, antibodies against flu can prevent adsorption of the virus to sialoconjugates by binding close to the sialic acid binding site, or they can bind to the stem region of the HA molecule and prevent its transition from the pre-fusion to the fusogenic state (Laursen & Wilson, 2013). Other antibodies have been found to have poor neutralizing capacities, but have been shown to demonstrate potent antiviral through their recruitment of immune effector function such as complement or immune cell recruitment. Other groups have engineered antibody conjugates in order to direct drugs to a particular cellular marker.

Antibodies' specificities towards their targets affords them the versatility to explore remarkable applications in therapeutics.

Here we report the identification of two anti-EBOV neutralizing VHHs. We evaluated their affinity and specificity for recombinant EBOV GP and demonstrate their ability to neutralize EBOV-pseudotyped VSV as well as Ebolavirus (Kikwit).

Results

Nanobodies that recognize EBOV GP and neutralize infection.

To generate VHHs specific for the Zaire Ebolavirus (EBOV) glycoprotein (GP), we immunized alpacas with preparations of Ebola virus-like particles (VLPs) consisting of EBOV GP and the major matrix protein (VP40) derived from Ebolavirus Zaire (Mayinga, 1976). After five rounds of immunization, we isolated peripheral blood lymphocytes from the immunized alpacas, extracted RNA, prepared cDNA, and amplified buy PCR the VHH coding sequences using conserved primers. The amplified VHH cDNAs were cloned into a phagemid vector to generate a phagemid library containing the immunized alpaca VHH library (**Figure 11**). We monitored the immune response against GP by performing an immunoblot against recombinant GP provided the lab of Sina Bavari (USAMRIID). Using unimmunized serum against post-immunization serum, we are able to see signal corresponding the EBOV GP in the post-immunization and not the pre-immunization serum, indicating the there was a specific immune response against the immunogen.

To isolate VHHs specific for GP, we immobilized recombinant GP on beads or tissue culture-treated 6-well plates and selected for GP-binding phage through 2 rounds of panning.

Two unique VHHs were identified which were then tested for their neutralizing ability in an infection assay.

Ebolavirus VHHs that neutralize VSV-EBOV infection

To test the ability of the VHHs to block Ebolavirus infection, we expressed them in *Escherichia coli* and purified them by means of their C-terminal His tag on Ni-NTA beads. Using Ebola GP-pseudotyped VSV, we then assessed their neutralization profile. The pseudotyped viruses express GFP when infection is successful and thus allows easy monitoring of infectivity by flow cytometry.

To identify VHHs capable of blocking infection, we incubated virus stocks with individual VHHs prior to their administration to cells. We found that two VHHs, designated VHH-G10 and VHH-G84, when mixed with VSV-EBOV, blocked infection of Vero cells (**Figure 13**). An irrelevant VHH (VHH68) against Influenza A hemagglutinin (IAV HA) was ineffective in this assay, establishing specificity. We normalized the extent of infection in the presence of VHHs against VHH68. VHHs G10 and G84 interact and neutralize VSV-EBOV infection **(Figure 13**), with infection being reduced by approximately 95% at a VHH concentration of 667 nM. Provided the neutralization profile, we found that the IC50 of G10 and G84 were 17.65 and 90.1 nM, respectively.

These VHHs were recovered through panning using recombinant GP as the bait. We next confirmed the specificity of interaction in a direct ELISA in which we immobilized recombinant GP lacking the mucin domain onto 96-well plates. We incubated VHHs site-specifically biotinylated at their C-terminus via a C-terminal sortase recognition site, LPXTGG, labeled with

GGG-biotin using sortase. This enabled the detection of bound VHHs using a Streptavidin-HRP adduct as the secondary reagent. G10 and G84 interact with GP with an EC50 = 110 and 390 pM, respectively (**Figure 14**).

To explore whether G10 and G84 bind to the same epitope on GP, we labeled the C-terminus of the testing VHHs with GGG-AlexaFluor647 (GGG-AGF647). To test for competition of binding, we co-incubated labeled VHHs with increasing concentrations of unlabeled VHH as a competitor. We found that there is competition between G10 and G84 (**Figure 14**) detectable signal (AF647) from flow cytometry diminishes in a dose-dependent manner, with approximately 50% of the signal being lost at 100 times molar excess of competing VHH, G84 (**Figure 14**).

V-regions derive from distinct clades

Previous work found that heavy-chain only V-regions derive some the same IgH locus, however the C_H regions utilized contain mutations allow for proper formation of heavy-chain only antibodies that no longer require interaction with a cognate C_H1 domain (Achour et al., 2008). In order to determine whether these VHHs are derived from the same germline, we looked at the sequence alignment using the IMGT deposited V-regions from *Vicugna pacos*. Determining if these VHHs derive from the same V-region may shed light if particular V-regions are predisposed to formation of specific neutralizing antibodies, as is the case for VRC-01 anti-HIV antibodies (West, Diskin, Nussenzweig, & Bjorkman, 2012).

By aligning the sequences to deposited IMGT sequences, we found that these VHHs cluster into two different subgroups as defined in (Achour et al., 2008). G10 derives from subgroup A, and G84 clusters with members of subgroup C (**Figure 12**).

G84 interferes VSV-EBOV infection after attachment has occurred

G10 and G84 clearly derive from 2 different germline V segments as inferred from the use of different germ-line encoded CDRs 1 and 2, as well as differences in the framework regions (**Figure 12**). Their CDR3s also differ. Both G10 and G84 neutralize VSV-EBOV but it is unclear whether they do so by a similar mechanism.

To determine whether there is a different function between the two VHHs, we developed an assay that would be able to bifurcate the two functionalities if inhibition is to occur at the level of attachment. In this case, we adsorbed virus onto the surface of cells while inhibiting internalization by chilling on ice. By following with a dosage of VHH expected to give full neutralization in solution, we found that G84 was still able to neutralize infection despite attachment occurring (**Figure 13**).

Neutralization of Ebolavirus

Antibodies that neutralize VSV-EBOV do not necessarily neutralize Ebolavirus. To assess whether our VHHs can also block EBOV, we worked with the lab of Christopher Cooper to assess whether our VHH neutralization profile can be recapitulated against Zaire ebolavirus.

To this end, we tested the ability for VHH-G10 and VHH-G84 to block the Kikwit, 1995 strain of Zaire Ebolavirus. In collaboration with USAMRIID, we wanted to assess whether our

observations with VSV-EBOV neutralization can be recapitulated with live Ebolavirus. VHHs G10 and G84 were co-incubated with the Kikwit strain of Ebolavirus and adsorbed to a monolayer of Vero cells. Cells were analyzed by high-content automated fluorescence microscopy by staining cells with DAPI and anti-GP IgG. By overlaying the DAPI and GP channels, we can determine the changes in infectivity in the presence of G10 and G84.

We chose to use the reference neutralizing antibody, 13C6, as a reference to compare the neutralizing capacity of our VHHs to a known neutralizing antibody. We carried out neutralization assay using half-log dilution series and assessed the extent of cellular infection. For example, at the highest concentration tested (100 ug/ml), both VHHs neutralize the Kikwit strain more effectively than 13C6 at the same mass concentration (**Figure 15**).

VHH G10 does not cross-react with distantly related Ebolavirus

In an effort to determine the epitope at which G10 binds, we collaborated with the Saphire Lab (Scripps Research Institute) to attempt to crystallize the VHH in complex with recombinant Zaire Ebolavirus (Mayinga, 1976) glycoprotein. While the efforts to form an intact crystal ultimately was unsuccessful, they were able to test whether G10 was able to recognize a closely related glycoprotein. The Bundibugyo strain of Ebolavirus is the next closest Ebolavirus strain to the Zaire strain, and G10 was unable to recognize this via ELISA (**Supplemental 4**).

As recombinant GP expression is increased significantly by removal of the large mucin-like domain, we also assessed binding to this variant (**Supplemental 5**). They found that removal of the MLD does not affect binding of G10. In addition, they assessed whether removal of the glycan cap, as is achieved by cathepsin cleavage affects binding. They found that there is

a possible effect on binding and that the glycan cap possibly stabilizes the interaction of G10 to GP.

In Progress

Investigation of the Mechanism of Neutralization

While our current data demonstrates that VHHs G10 and G84 both neutralize infection of VSV-EBOV, our current understanding is limited by only functional experiments. We currently lack a mechanistic understanding of neutralization. Our experiments have found that G10 and G84 neutralize infection differently (**Figure 13**). GP function is activated upon cathepsin B or L cleavage, allowing for engagement to NPC1. Once NPC1 engagement occurs, insertion of the internal fusion loop facilitates release of the host genome into the host cell. Thus, we will be exploring the mechanism of neutralization through inhibition of these three host factors.

Cathepsin-Mediated Inhibition

VHH-G84 has been demonstrated to neutralize infection after adsorption has already occurs. This observation suggests that G84 is interfering with one of the many intracellular factors that Ebolavirus GP interacts with in order to conclude with successful fusion. As such, one of the first entities that GP interacts with upon endocytosis is with endosomal resident proteases, cathepsins B and L. Here we will be utilizing a technique similar to protease foot-printing to determine whether cathepsin activation is inhibited by the presence of VHHs G10 and G84.

Experimental Setup:

We have developed a system in which we have C-terminally labeled the Ebolavirus glycoprotein with a C-terminal 3X FLAG tag in pWRG7077. This results in surface expression of GP that can be immunoprecipitated using a monoclonal antibody against the FLAG tag. Thus, we can track the fate of expressed GP in different conditions using immunoprecipitation. Cathepsins B and L's ability to proteolytically cleave GP in the presence of G10 and G84 will be assessed.

Expected Results:

In order to accurately monitor GP expression, we first looked at the expression profile of GP over time. To do this, we biosynthetically labeled 293T cells that have been transiently transfected with pWRG7077 EBOV-GP-3XFLAG. Using an anti-FLAG (M2) conjugated sepharose, all expressed GP can be precipitated using its C terminal tag. As we have designed the C terminal to tag to the transmembrane portion, precipitation of soluble GP is not possible (Dolnik et al., 2004). The observed profile can be seen in **Figure 16.**

In order to determine whether the VHHs play a role in inhibiting cathepsin mediated proteolysis, we designed as assay that would be able to determine if VHHs interfere with the kinetics or recognition of the cathepsin cleavage motif. To do this, we will perform a biosynthetically label cells that express Ebolavirus (Mayinga, 1976) glycoprotein and assess whether cathepsin B and L cleavage is inhibited. We will vary the duration of cathepsin exposure. We will also incubate the VHHs with the precipitated GP prior to cathepsin activation. If inhibition occurs, we should see deviations in the proteolysis profile in the presence and absence of VHH.

To monitor whether GP expressed on the surface of the cell can act as a substrate for purified GP, we subjected precipitated GP to cathepsin digestion. In order to carry out this experiment. In this case, we subjected cells to chasing for 4 hours, after a 1 hour pulse to ensure accumulation of the glycoprotein. We simultaneously activated cathepsins B and L by incubating them in a 100 mM MES, 5 mM DTT at pH = 5.0 for 15 minutes at room temperature. Reactions proceeded at 25C and quenched by adding boiling loading buffer without DTT. Samples were subsequently boiled for 10 minutes.

Ebolavirus GP expression can be visualized using pulse chase analysis (**Figure 16**). As expected the synthesis of transiently expressed GP in HEK 293T cells follows what is expected of proteins that are translated through the ER and undergo sequential maturation steps such as post-processing events such as furin-like protease cleavage as well as glycosylation in the Golgi.

Preliminary data suggests that G84 may interfere with cathepsin B proteolysis of membrane bound GP as indicated in **Figure 17**. For this experiment, cells were pulsed for 1 hour before chasing for 4 hours. After anti-FLAG immunoprecipitation, GP precipitates were either exposed for 100 mM MES, pH 5.0 supplemented with fresh 5 mM DTT in the presence or absence of 10 μg/ml human cathepsin B for the indicated time points. Reactions were quenched with the addition of boiling SDS loading buffer. Here we see a lower intermediate (GP1*) formed during proteolysis of VHH68 and G10, but not in the presence of G84. A 50 kDa intermediate was observed during proteolysis using cathepsin B (Schornberg et al., 2006). GP1* may represent this species as appearance of it coincides with increased intensity at 25 kDa (**Figure 17**). This would suggest that G10 does not prevent cleavage, G84 is able to perturb cathepsin B cleavage.

NPC1-mediated Neutralization

The importance of Niemann-Pick C1 (NPC1) in viral entry is paramount (Carette et al., 2011). As exposure of the receptor binding site on GP is facilitated by the proteolytic activation by cathepsins, it is conceivable that G84 may interfere with NPC1 engagement and thus prevent fusion from occurring. In order to determine whether the VHHs interfere with NPC1 engagement, as with mAb-548 (Wec et al., 2016). In order to test this hypothesis, we will be designing a multi-pronged approach in order to address this possibility.

Experimental Design

First, we will generate a NPC1 KO Vero in order to generate cells that are no longer susceptible to Ebolavirus infection via CRISPR/Cas9 to introduce an insertion or deletion in the first exons of NPC1. Using a NPC1 expressing plasmid, we can demonstrate rescue of infection and protection once VHHs are present. Using these NPC1 KO cells, we can also express a tagged version of NPC1 in order to co-immunoprecipitation NPC1 with GP. We have also received CHO-M12 NPC1 knockouts and constitutively expressing human NPC1 from the Cunningham Lab, HMS. We will be performing the same experiments as described above.

In collaboration with the Kirchausen lab, we will be observing the fate of neutralized virus in the presence of neutralizing virus. Using a NPC1-TagRFP cell line, we can track virus inside of a cell. For this experiment, we will be using VSV-M-eGFP EBOV. This variant of the pseudotyped VSV does not express GFP upon infection; instead, it expresses a genetic fusion of the M protein with eGFP. In this way, VSV-EBOV particles eGFP labeled and trackable by spinning disk confocal microscopy or lattice light sheet microscopy. In addition to NPC1, we will

also be tracking endosomal compartments using Rab5, Rab7, AP2, and other cell biological fusion proteins of interest in order to monitor the fate of VSV-EBOV in various compartments as they proceed through the various stages of their infectious life cycle.

The questions we hope to address with this collaboration are: 1) how does G10 or G84 affect internalization 2) is the fate of endosomal trafficking affected either on a kinetic or endpoint level? 3) how far do viruses internalize from the cellular membrane? We will be performing pilot experiments using AP2-tagRFP and eventually expand into other relevant cell biological markers.

Expected Results

We have demonstrated that CHO-M12 NPC1 knockout cells are unable to be infected by VSV-EBOV. Consequently, the ability for VSV-EBOV to infect these cells are restored when they have been transiently transfected with pcDNA3.1 NPC1-FLAG (Genscript) in a dose-dependent manner.

To determine whether G10 and G84 perturb the interaction of NPC1 and cleaved GP, we will observe whether we can see the co-localization of tagged NPC1 and the virus. We have also modified the C-terminus of the VHH, therefore we can assess whether the lack of engagement of NPC1 to GP was a consequence of the VHH potentially inhibiting the interaction or whether the lack of interaction was not related.

In addition, we will also transfect NPC1 null CHO cells with an overexpression plasmid that will be able to restore infection of VSV-EBOV. If the VHH block the interaction of NPC1 to GP, we should be unable to recover GP in the presence of VHH.

Discussion

Since its discovery in 1976, Ebolavirus is still a devastating infectious disease that affects Western Africa. The deployment of ZMapp quickly illuminated the need to develop further strategies to treat individuals effectively. VHHs are emerging as a promising alternative to conventional antibodies for passive immunization treatment strategies. Conventional antibodies are not as stable in warm environments (Vermeer & Norde, 2000). VHHs exhibit higher thermostability, thereby eliminating the need for refrigeration.

Vaccine development against a number of viral pathogens has been remarkably successful, but with limited success for Ebolavirus so far. Infection with Ebola remains a threat, requiring a greater selection of antibodies for post-exposure prophylaxis and for passive immunization. We describe two VHHs, derived from alpacas immunized with Ebola virus-like particles that can neutralize infection *in vitro*. We found that two VHHs, G10 and G84 interfere with infection by two different mechanisms. We know of no other VHHs that neutralize Ebolavirus infection.

A number of conventional antibodies have been identified that can neutralize infection *in vitro*, but apparently do not confer protection *in vivo (Gunn et al., 2018)*. Ebola GP, the viral protein essential for entry, is the primary target for the current generation of neutralizing antibodies. The presence of an Fc is important to confer protection (Gunn et al., 2018), especially for antibodies that lack the ability to neutralize *in vitro*. VHHs lack an Fc portion and are monovalent. VHHs that can bind to Ebolavirus GP have been identified, however, no

additional functional experiments carried out to determine their ability to neutralize (J. L. Liu et al., 2017).

The monovalent nature of VHHs presents itself as an opportunity to dimerize VHHs through different avenues. By appending an Fc portion, generation of VHH dimers can be achieved with the ability to interchange different Fc portions for desired effector activity (Gunn et al., 2018). Click-chemistry affords the ability to generate C-to-C dimers by addition of C-terminal click handles to the VHHs, allowing for control of the generation of homo- and hetero-dimers without additional effector function (Witte et al., 2012). By generating tandem VHHs, the control the functionality, avidity, and specificity can be fine-tuned for the desired purpose. For example, a bispecific antibody that targeted both the Ebolavirus GP and NPC1 was utilized to perturb the required interaction of NPC1 engagement (Wec et al., 2016). Targeting multiple epitopes of the same target or to different targets would indeed be a useful approach to tackle viral neutralization.

The role of Fc in protection is indeed being more appreciated as a number of antibodies that have poor neutralization profiles *in vitro*, appear to be necessary for robust protection in an *in vivo* mouse or NHP infection models (Gunn et al., 2018). VHHs do not have effector function and do not require interaction with a compatible light chain. Thus, appending the appropriate F portion for the desired activity, such as complement activation or natural killer (NK) cell recruitment, tailoring of the VHHs effector functionality can be easily achieved. In addition, our screen identified 15 different VHHs against Ebolavirus, however, the remaining 13 did not neutralize infection as robustly as G10 or G84 (data not shown). However, these VHHs show the mutations in FR2 that would identify them as derivatives of the heavy chain only

antibody (**Supplemental 2**). As Fc function has not yet been explored, attachment of F portion to these VHHs can expand the functionality in animal studies as well as cytotoxicity assays.

Our study has not yet addressed the mechanistic properties of neutralization; however, EBOV-GP has been well characterized in terms of functional epitopes present on the glycoprotein surface. As G84 function interferes with function once internalization as proceeded, it is possible it functions through the inhibition of cathepsin B and L or blockage of the NPC1 engagement (A. L. Rasmussen, 2016). In particular, as the contribution of the Fc portion towards *in vivo* protection is , the versatility at which to tailor VHH-based inhibitors are limited by our imagination as Ebola vaccines are able to provide robust preventative treatments.

Materials and Methods

Immunization

Alpacas were immunized by administration of Ebolavirus VLPs provided by USAMRIID. Two alpacas were immunized 5 times with a VLPs. A VHH plasmid library was generated by isolation of RNA from peripheral blood lymphocytes to use as a template for cDNA library generation. We used 3 primer sets (random hexamers, oligo dT, and primers specific for the constant region of the alpaca heavy chain). VHH coding sequences were amplified using VHH specific primers and cut with AscI and NotI and ligated in pD (phagemid vector). E. Coli TG1 (Agilent) cells were electroporated with the ligation reaction and ampicillin-resistant clones were selected, harvested, and stored as glycerol stocks.

Bio-panning

EBOV-GP specific VHHs were obtained by phage display and panning with a protocol. E. coli T1 cells containing the VH library were infected with helper phage VCSM13 to produce phages displaying the VHHs as pIII fusion proteins. Phages in the supernatant were purified and concentrated by precipitation. Recombinant GP was either biotinylated using NHS-biotin (ThermoFisher) or immobilized onto tissue culture 6-well plates (Corning). For panning using biotinylated GP, Streptavidin T1 Dynabeads (Life Technologies) was used to enrich for VHHs specific for EBOV-GP. Obtained phages were used to infect E. coli ER2738 and subjected to a second round of panning. ER2837 colonies were diluted such that individual colonies could be cultured individually in 96 well plates. VHH expression was induced using IPTG. Recombinant GP was immobilized onto high affinity 96-well plates (Corning) and bound VHHs were detected using HRP coupled to anti-E tag antibodies (Bethyl Laboratories). Specificity was monitored using a non-specific VHH, VHH68 against anti-Influenza HA.

Expression of single domain antibody fragments

Expression and purification was previously described in (F. I. Schmidt, L. Hanke, et al., 2016). Briefly, VHHs were cloned into a pHEN6 backbone that allows for periplasmic expression of VHH in WK6 *E. coli*. WK6 was grown to OD600 = 0.8, and induction was initiated using 1 mM IPTG. VHH expression induction continued at 30C overnight. VHHs were purified using Ni-NTA, followed by size-exclusion chromatography using a Hi-Load SuperDex 75 (GE) column.

Generation of VSV-EBOV/GFP

The lab of Sean Whelan (HMS) generously provided VSV-EBOV-GP (Mayinga, 1976) seed stocks. VSV-EBOV was produced by transduction of BsrT7 (BHK, stably expressing T7

polymerase) for 24 hours. Supernatants were harvested and spun at a low speed to remove cellular debris. Viral supernatants were further purified through a 10% sucrose in NTE cushion by ultracentrifugation. Virus pellets were resuspended in NTE overnight at 4C. When it is necessary to remove defective viral particles, viral preparations were further purified through a 10 – 45% sucrose-NTE solution. Viral suspensions were snap frozen prior to storage in -80C.

Viral titers were calculated by seeding Vero cells. Viral inoculums were diluted and adsorbed to cells for 1 hour at 37C. Inoculum was removed and fresh media was added; cells were incubated for 16 hours. Cells were fixed and analyzed by flow cytometry for GFP expression. Titers are reported as FFU (foci forming units). Typical titers were ~ 10^8 FFU/ml.

In Vitro Neutralization

Vero cells were seeded into 24 well treated plates. Virus (final MOI = 1.0) was co-incubated with different concentrations in DMEM (Corning) in the absence of cells at 37C for 1 hour. After incubation of VHH: virus inoculum, growth media was aspirated and replaced with virus inoculum, incubating for 1 hour at 37C with gentle rotation every 15 minutes. After 1 hour, inoculum was aspirated and replaced with complete growth media and cells were grown overnight. In preparation for flow cytometry, cells were trypsinized in 0.05% trypsin-EDTA (Life Technologies) and washed with cold PBS once. Cells resuspended and fixed in 4% formaldehyde for 20 minutes at room temperature.

For FACS analysis, we quantified GFP-expressing cells and normalized against a non-specific VHH (anti-influenza HA) control. All samples were analyzed using BD Accuri C6.

Post attachment neutralization

Vero cells were seeded into 24 well treated plates. Inoculation of VSV-EBOV was performed at a MOI = 1.0. Infections were performed by pre-chilling cells for 5 min on ice. Virus as adsorbed onto cells on ice for 1 hour. Cells were washed once with cold PBS once. VHHs G10 and G84 diluted in DMEM were added at 200 nM for 1 hour on ice, then incubated for 1 hours at 37C. After incubation, inoculum was removed and infection was analyzed by GFP expression via flow cytometry after 16 hours.

Sortase Labeling

C-terminal modification of VHHs was performed by incubating VHH with 5 molar excess of GGG-biotin and sortase (hepta-mutant) in 50 mM Tris 7.4, 150 mM NaCl for 2 hours at RT. In the case of AlexaFluor647 (AF647), VHHs were incubated in 1.5 molar excess GGG-AF647 overnight at 4C to minimize hydrolysis products. "Sortagged" VHHs were separated from unreacted VHHs by incubation with Ni-NTA agarose (Qiagen) for 1 hour, 4C. Supernatant was collected and buffered exchanged into PBS using PD-10 columns (GE). Excess probe is removed by spin filtration using a 5 or 10 kDa spin column (Amicon). Purity was confirmed through LC-MS and SDS-PAGE.

ELISA

100 ng of protein was immobilized onto Costar-treated high affinity plates (Corning 9018) overnight at 4C. Plates were blocked with 5% BSA in PBST (0.1%) for 2 hours. VHH were diluted into PBST + 5% BSA and incubated at RT for 1 hour. Streptavidin HRP was added for 1 hour, RT. Plates were washed with PBST 10 times. TMB (Sigma) was added and incubated at 37C for 30 min. Reactions were quenched with 1M HCl and signal was quantified by 562 nM.

Sequence Alignments

Sequence alignments were aligned and visualized using Jalview (GPL).

Biosynthetic Labeling

6cm plates were pre-treated with a 0.1% poly-L-lysine solution (Sigma #P8920) and dried overnight. 293T cells were seeded overnight. Seeded 293T cells were transfected with pWRG7077 expressing Ebolavirus GP, Mayinga 1976 with a C-terminal 3X FLAG Tag. 24 hours later, cells are biosynthetically labeled with 35-S.

Prior to pulsing, cells were starved in DMEM without methionine and cysteine for 15 minutes. Cells were pulsed with 110 uCi ^{35}S (EasyTag Express 35S, Perkin Elmer) per 2 million cells for 5 minutes or 1 hour. For longer pulses, 10% dialyzed FBS was added. Cells were subsequently chased with complete media containing cysteine and methionine. Cell synthesis was quenched by addition of PBS supplemented with 25 mM NEM for 10 minutes on ice. Cells were lysed using a solution containing 20 mM MES, 30 mM Tris. 100 mM NaCl, 1 mM EDTA with 1% Triton X-100 (MNT + 1% TX100) supplemented with 10 mM PMSF and HALT Protease Inhibitor Cocktail (Thermo Scientific). Soluble lysate was obtained and prepared in reducing and non-reducing conditions.

Immunoprecipitation

For C-terminal 3X FLAG constructs, anti-FLAG M2 Affinity Gel (Sigma-Aldrich) was resuspended in PBS with 0.25% BSA at a 10% suspension. 50 ul of beads was added to lysates and incubated overnight at 4C. IPs were washed with MNT at 25C. IPs were eluted using SDS Loading buffer at boiled for 10 minutes.

Samples were run on 12% SDS-PAGE and treated with PPO, diphenyl oxazole, in preparation for fluorography.

Cathepsin Proteolysis

Cathepsin B (Athens Research) was first activated in 100 mM MES at pH 5.0 supplemented with 5 mM DTT for 15 minutes at 25C at a concentration of 10 ug/ml. Activated cathepsin B was added directly to dried GP precipitants and incubate at room temperate for the indicated times, shaking at 1300 RPM. Reactions were quenched with boiling 4X loading buffer and immediately boiled for 10 minutes. Samples were prepared in non-reducing and reducing conditions supplemented with NEM.

Cell Culture

Cells were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin, unless otherwise indicated. All cells were grown at 37C and 5 % CO_2.

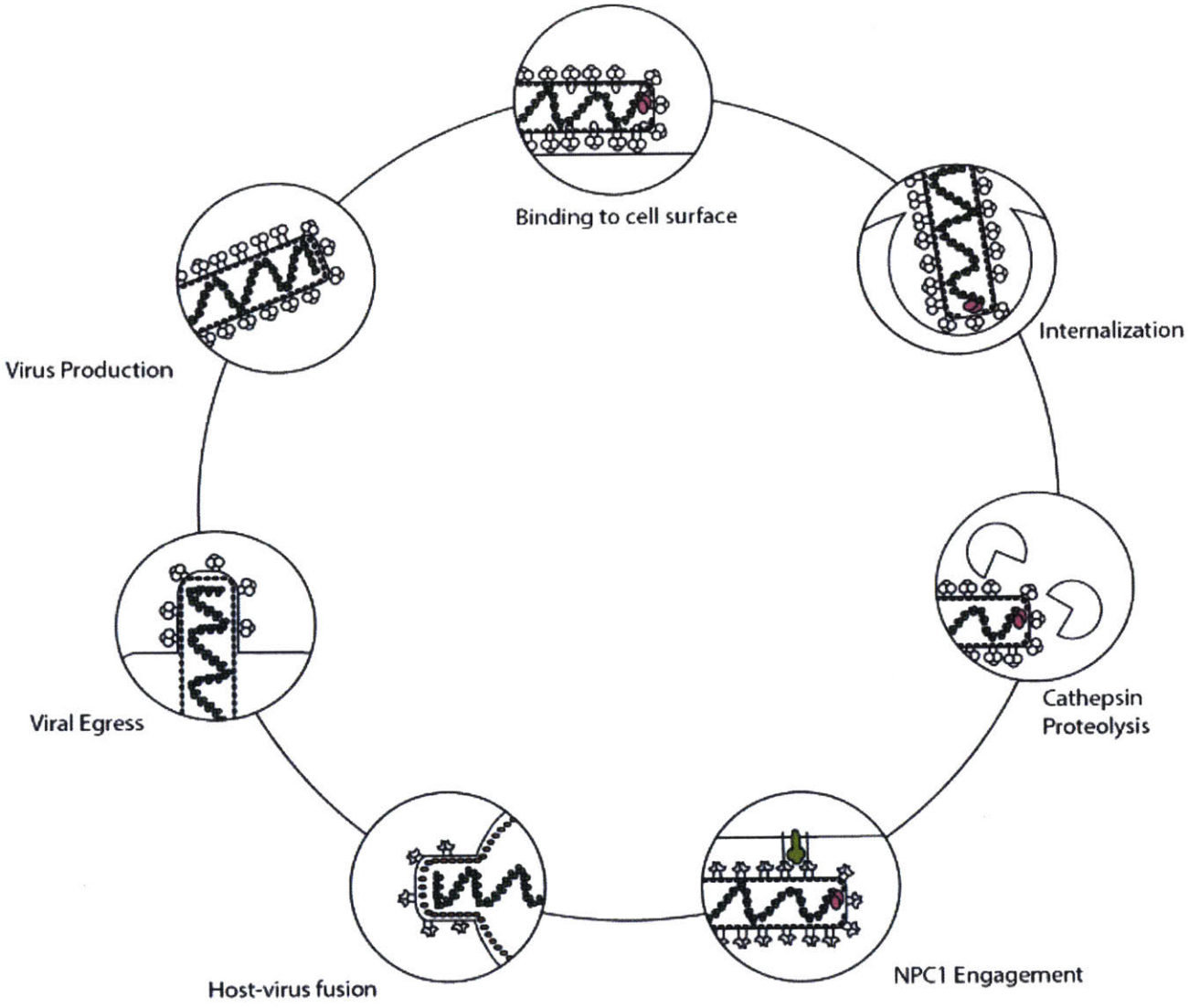


Figure 9 Ebolavirus Glycoprotein-Mediated Entry

The Ebolavirus glycoprotein is a multifaceted protein that mediates binding to the cell surface through currently unknown means. Internalization through macropinocytosis traffics he virus into endosomal compartments where it encounters endosomal resident proteases, cathepsin B and L. Proteolysis allows for GP to engage with NPC1 leading to viral fusion with the host membrane, releasing its genome to transform the cell into a viral factory. Virions are produced and released from the cell, ready to infect neighboring cells.

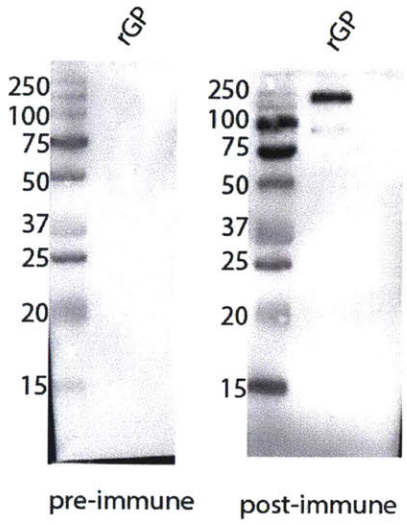


Figure 10: Immune response to GP is seen immunized alpaca serum

Pre- and post-immune serum from harvested from two alpacas that were immunized with Ebolavirus (Mayinga, 1976) virus-like particles consisting of the Ebolavirus glycoprotein and the major matric protein, VP40. Recombinant GP was blotted for using the serum and probed with anti-llama HRP (Abcam)

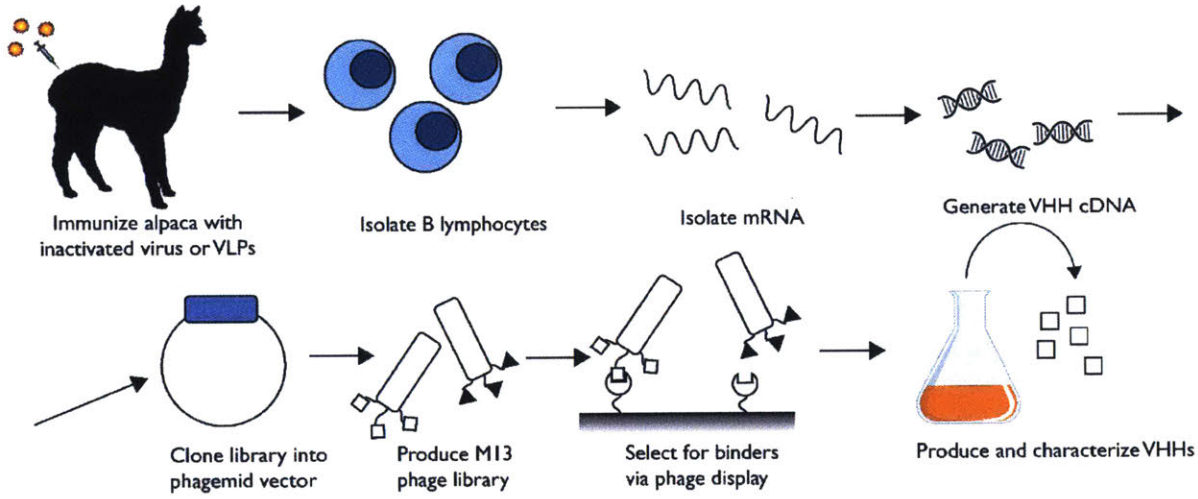


Figure 11: Schematic of VHH isolation from alpaca immunizations

2 alpacas were immunized with a suspension of Ebola virus-like particles 5 times. B lymphocyte cDNA libraries were isolated to generate a library of VHHs sing conserved primers to amplify the VHH encoding regions. These segments were cloned into a phagemid vector and used to produce M13 phage to isolate binders of VHHs specific to the Ebolavirus glycoprotein.

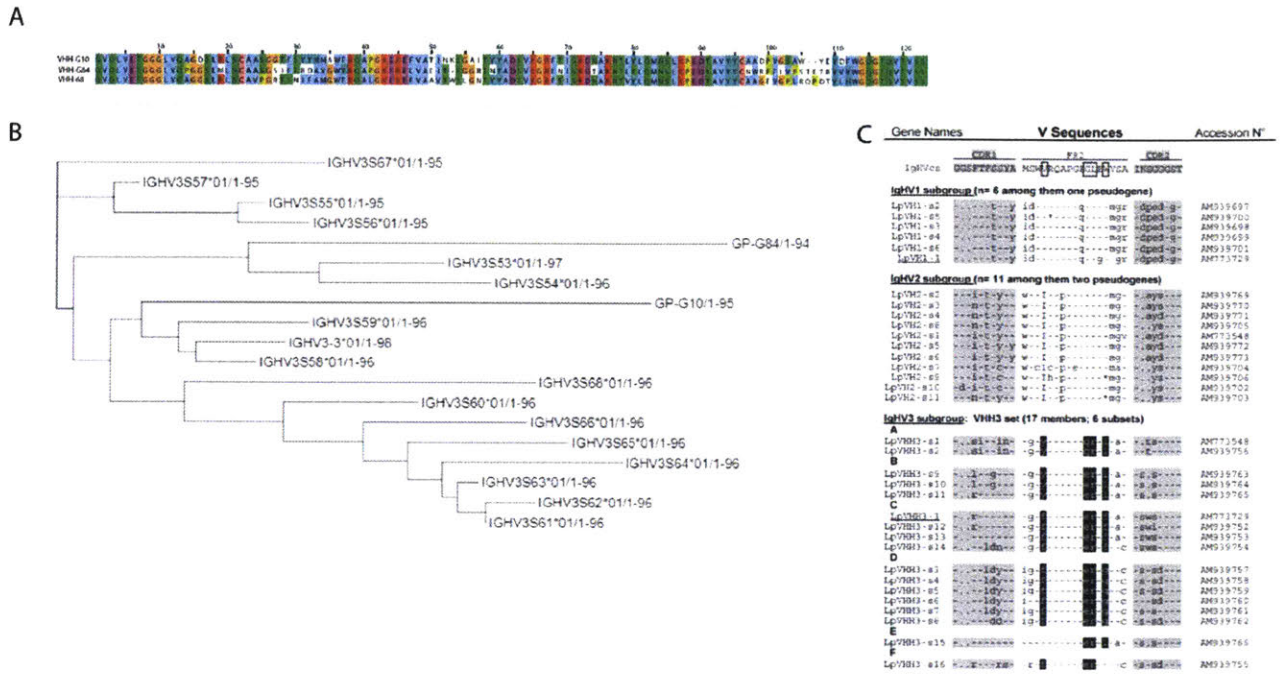


Figure 12: Sequences of anti-Ebolavirus Single domain antibody fragments

(A) G10 and G84 V region alignments were aligned using ClustalO alignment algorithm ad visualized using JalView. (B) G10 and G84 sequences were constructed using phytoT/iTOL. Sequences of germline V-regions were obtained using the IMGT database from Vicugna pacos (alpaca) C) Figure adapted from Achour et. al. 2001. JI

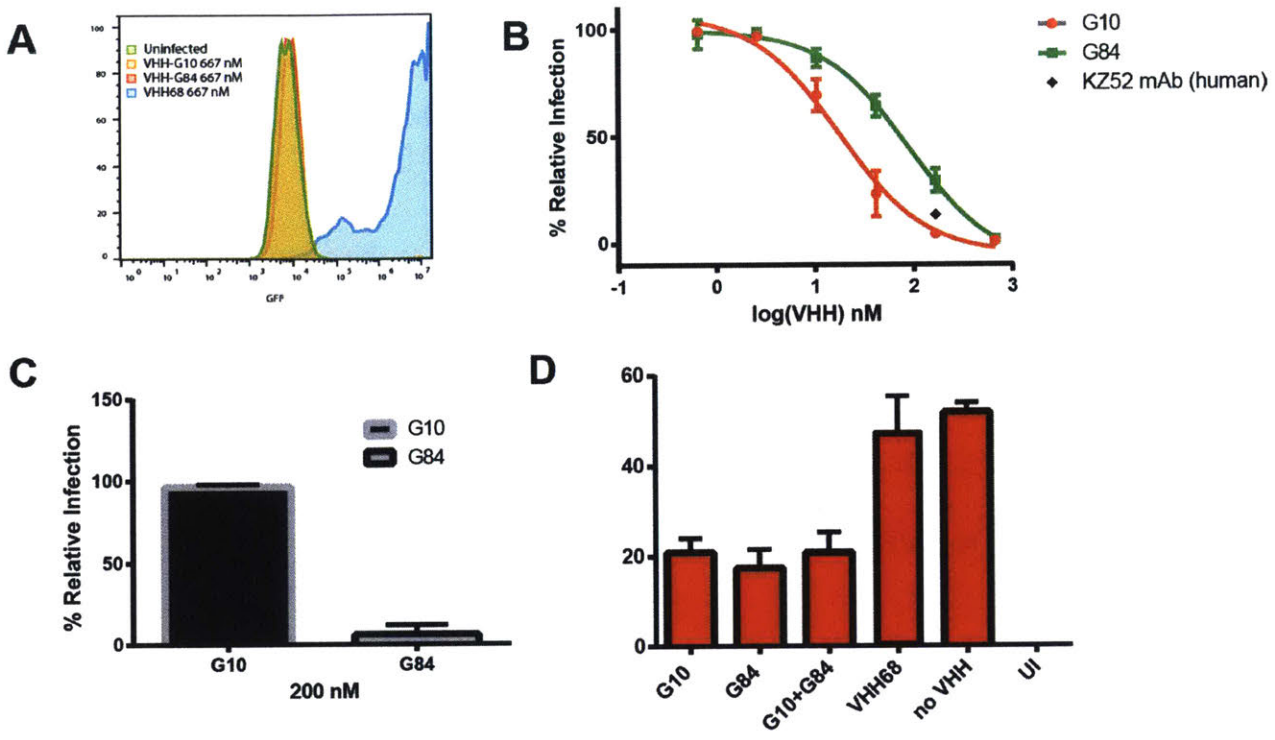


Figure 13: Neutralization of VSV-EBOV in cell culture

A) VSV-EBOV (MOI = 1.0) was added onto a monolayer of Vero cells and incubated overnight. Flow-cytometry was used to quantify infection via GFP. B) VSV-EBOV (MOI = 1.0) was incubated with G10 or G84 or KZ52. Virus-VHH inoculums were added to a monolayer of Vero cells and infection was scored by GFP expression via flow cytometry. C) VSV-EBOV (MOI = 1.0) was adsorbed onto the surface of a monolayer of Vero cells on ice. VHHs (200 nM) were added to cells after initial adsorption step on ice. Cells were brought to 37C and analyzed for GFP expression via FACS. D) Corresponding VHH combinations (total = 100 nM) were mixed with VSV-EBOV (MOI = 0.1) and added to cells as described in 3A.

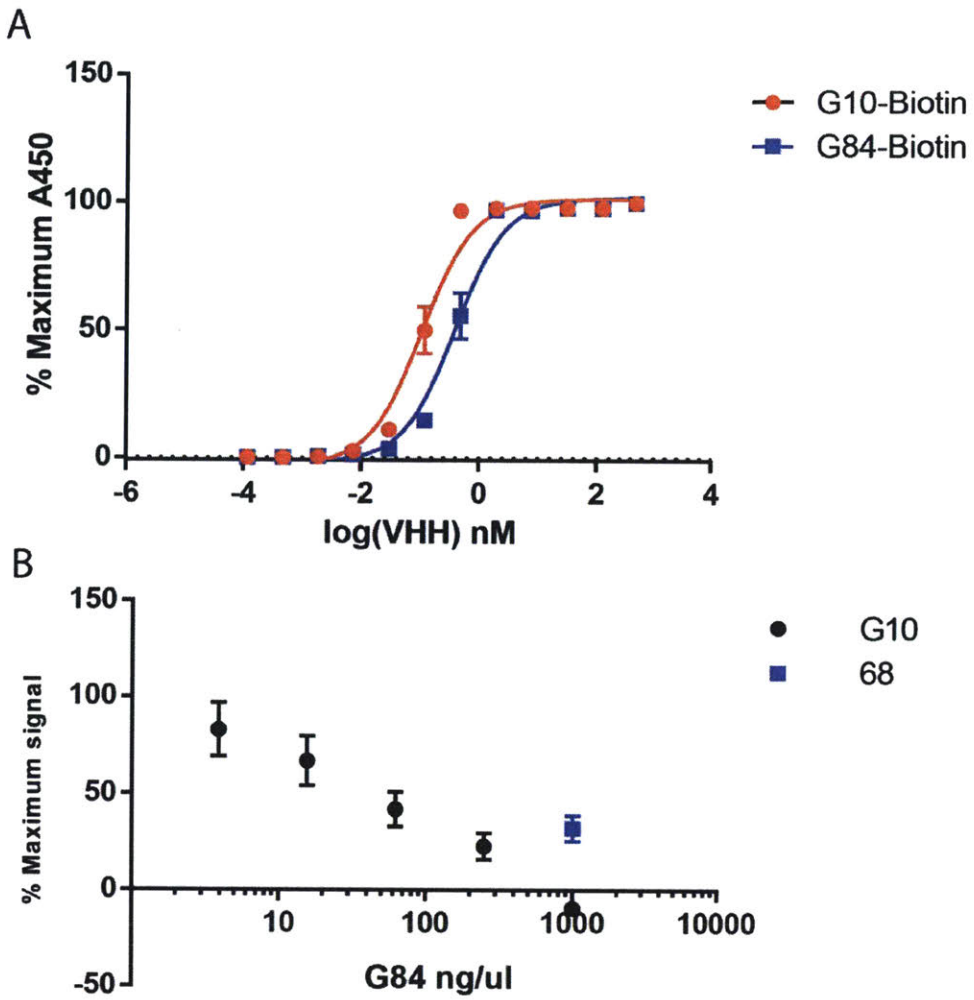


Figure 14: EC50 of G10 and G84 against recombinant GP lacking the mucin domain (GPdM)

Treated ELISA plates were adsorbed with 100 ng of GPdM. Using VHHs sortagged with a C-terminal biotin at different concentrations, the A450 was measured by TMB turnover after 30 minutes. Background signal was subtracted using VHH68 (anti-HA) as a non-specific VHH. B) VSV-EBOV infected cells were mixed with a corresponding concentration of unlabeled G84 and 1 ng/ul G10-AF647 for 1 hour on ice. Populations were gated to include only single, infected (GFP+) cells. Signal was normalized to a positive control in which no G84 competing VHH was present.

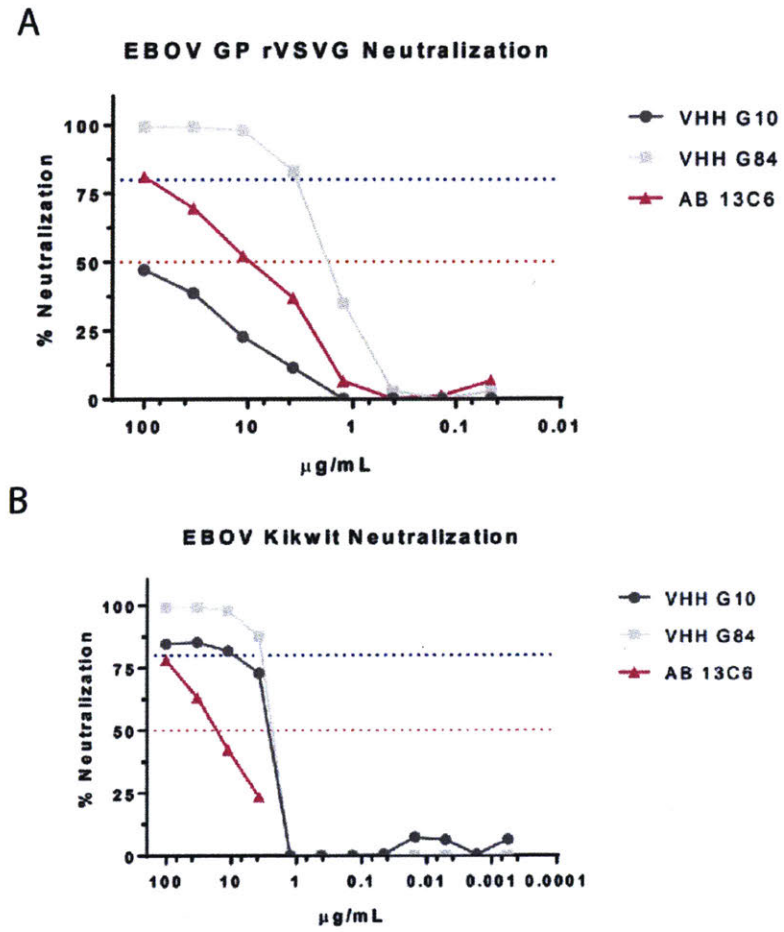


Figure 15: USAMRIID RIID Test- BL4

A) Recombinant VSV-EBOV was co-incubated with the indicated concentration of VHH. Infected cell were scored through co-localization of the anti-GP and DAPI channels. Infection was normalized using a non-specific VHH, VHH68. 13C6 was used as positive control neutralizing antibody. B) EBOV Kikwit were carried out similarly to recombinant VSV-EBOV infection.

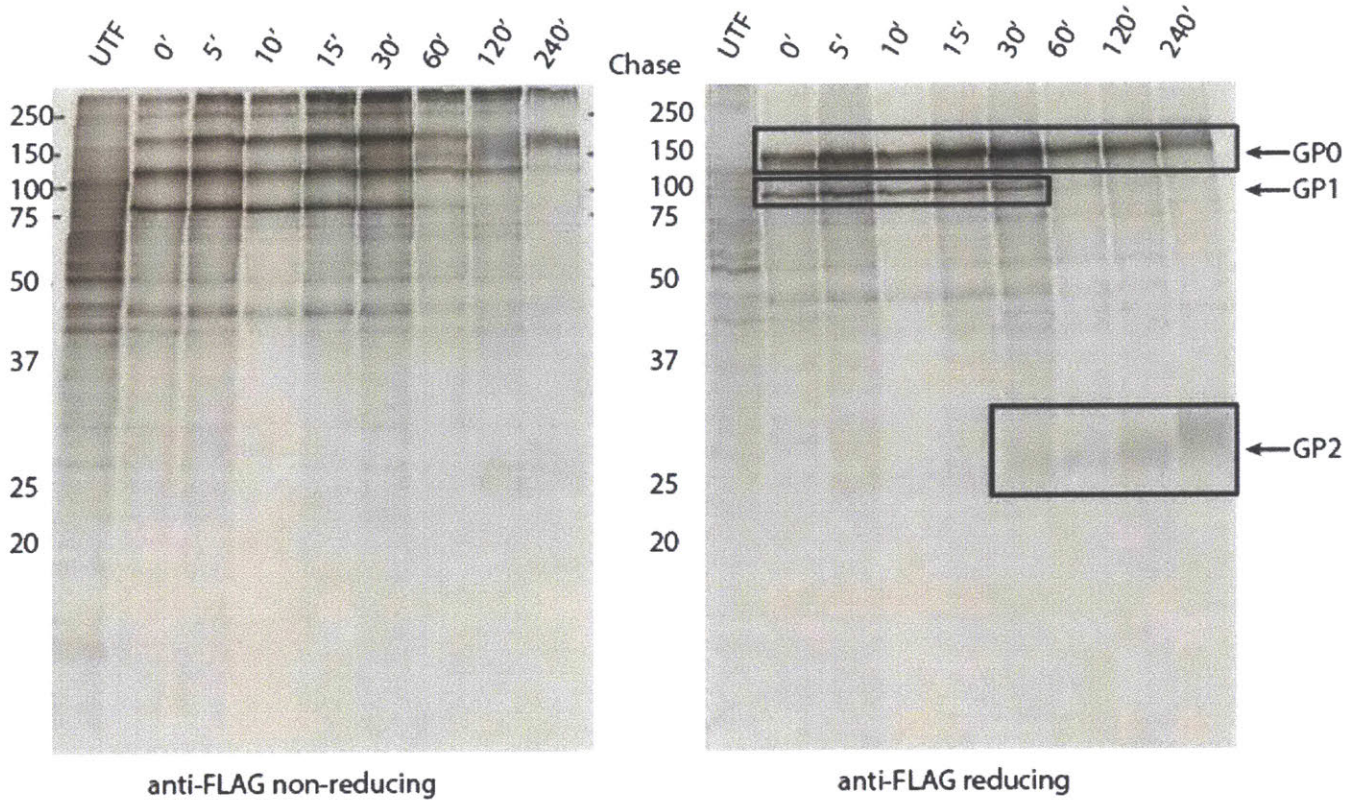


Figure 16: Expression of Ebola GP

293T cells were seeded onto poly-L-lysine treated plates and pulsed with 110 uCi 35-S. Ebolavirus GP was immunoprecipitated using M2 anti-FLAG sepharose (Affinity Gel, Sigma). Gels were run in non-reducing and reducing conditions in the presence of NEM.

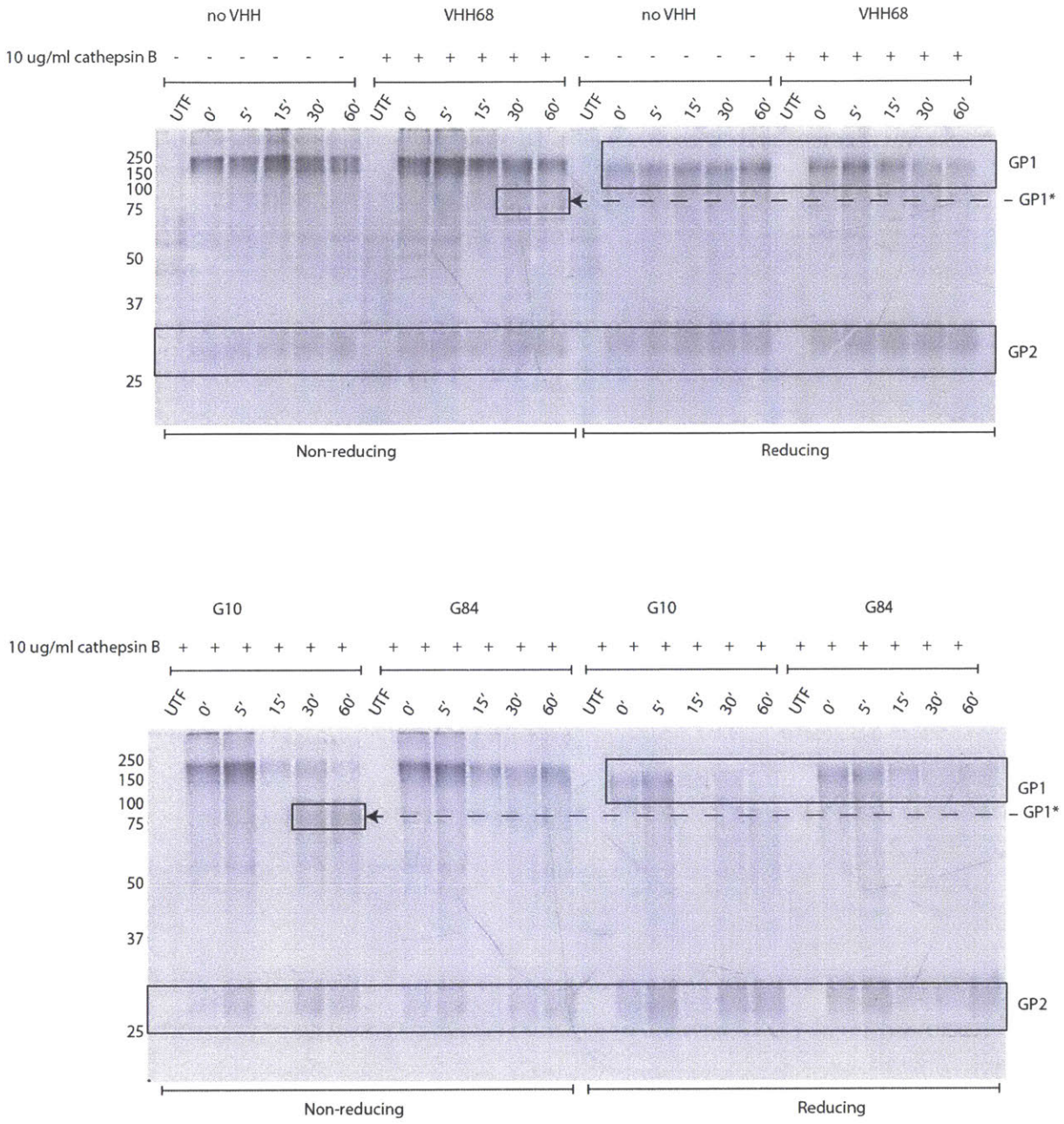
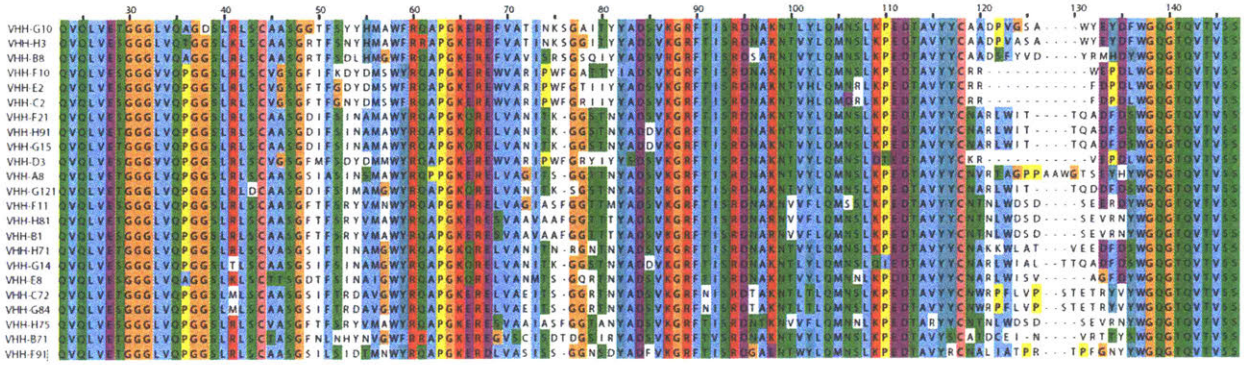


Figure 17: Cathepsin B Cleavage is altered by G84

293T cells were transfected with pWRG7077-EBOV GP. Transfected cells were biosynthetically labeled for 1 hour and chased with complete growth media for 4 hours. Full length GP was immunoprecipitated using anti-FLAG sepharose and subjected to cathepsin B (10 ug/ml) cleavage for the indicated times.

Supplemental Figures

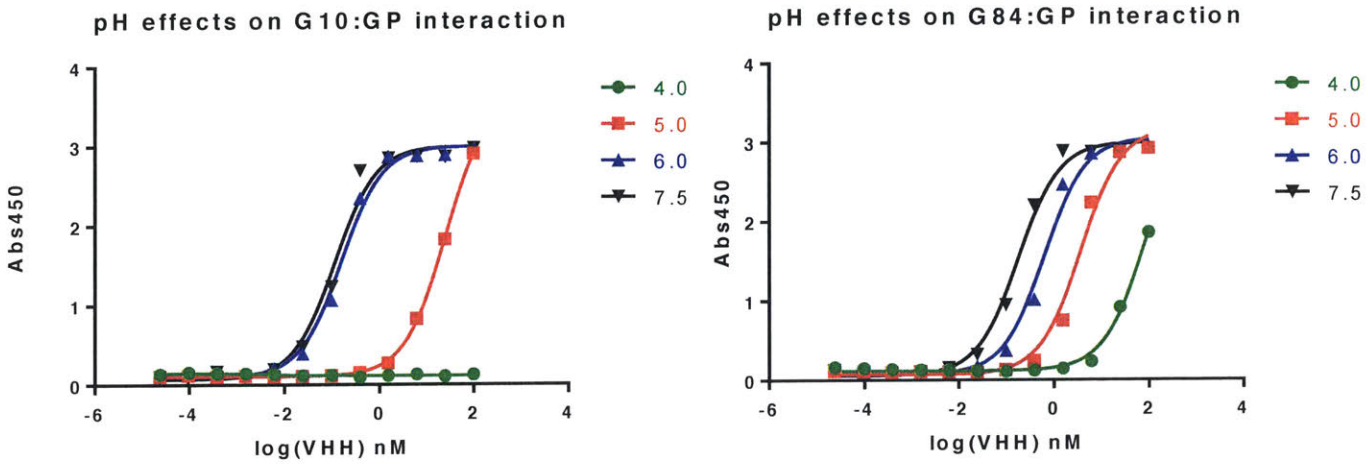


Supplemental 1: VHH Sequences

	CDR1	FR2	CDR2
Cons	GGSFTFSSYA	MSWVRQAPGKGLEWVSA	INSGGGST
G10	GG--TFSYYH	MAWFRQAPGKEREFVAT	INKSGAIT
G84	GSIFT--RDA	VGWYRQAPGKERELVAE	I-TSGGRT
H3	ASGRTFSNYH	MAWFRRAPGKEREFVAT	INKSGGIT
F10	GSGFIFKDYD	MSWYRQAPGKEREWVAR	IPWFGATT
C2	GSGFTFGNYD	MSWFRQAPGKEREWVAR	IPWFGRII
E2	GSGFTFGDYD	MSWFRQAPGKEREWVAR	IPWFGTII
D3	GSGFMFSDYD	MMWYRQAPGKEREWVAR	IPWFGRYI
B8	ASGRTFSDLH	MNWYRQAPGKERELVAG	IPWFGATT
F11	ASGFTFSRYV	MAWFRQAPGKEREFVAT	IASFGGTT
E8	TSGDTFSINA	MAWYRQPPGKERELVAG	MTSGQRTN
A8	ASGSIASINS	MAWYRQPPGKERELVAG	ITS-GGTT
B1	ASGFTFSRYV	MAWYRQAPGKERESVAA	VAAFGGTT
F21	ASGDIFSINA	MAWYRQAPGKQRELVAN	ITKGGSTN
G14	ASGSIFSINA	MGWYRQAPGKQRELVAN	ITKGGSTN
H91	ASGDIFSINA	MAWYRQAPGKQRELVAN	ITKGGSTN

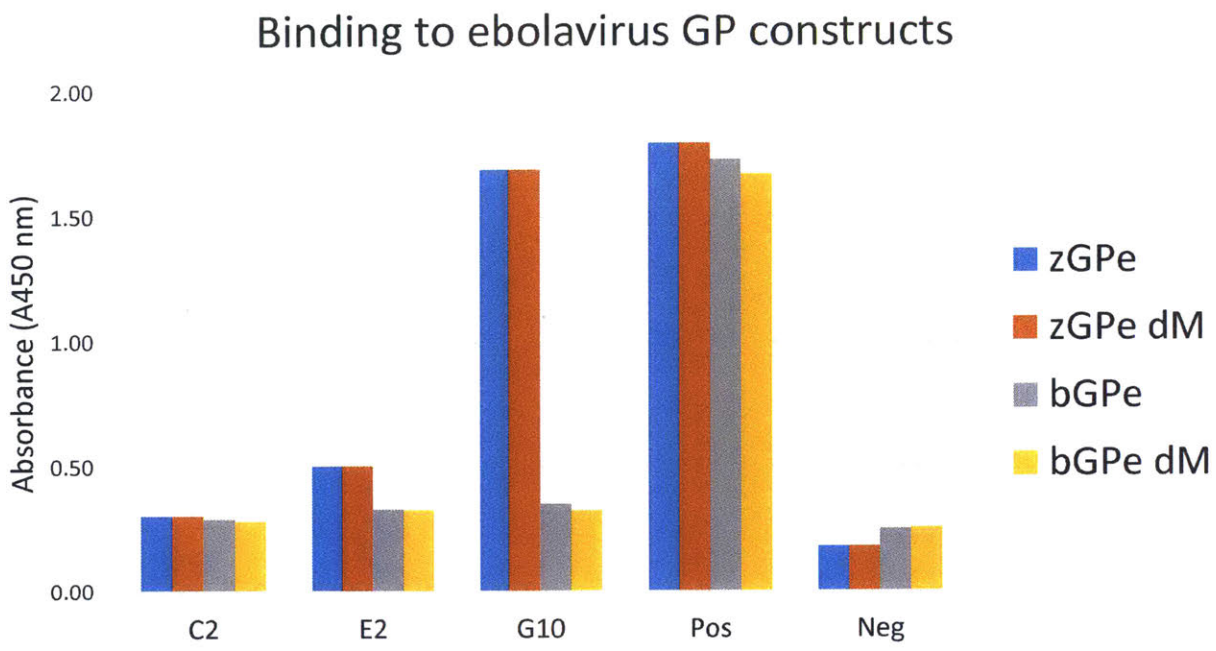
Supplemental 2: Analysis of the germline-encoded V-region of selected VHHs

Selected VHHs have diverse CDR1 and CDR2. Hydrophilic mutations in positions 37, 44, 46, and 47 indicate that these VHHs no longer require interaction with light chain.



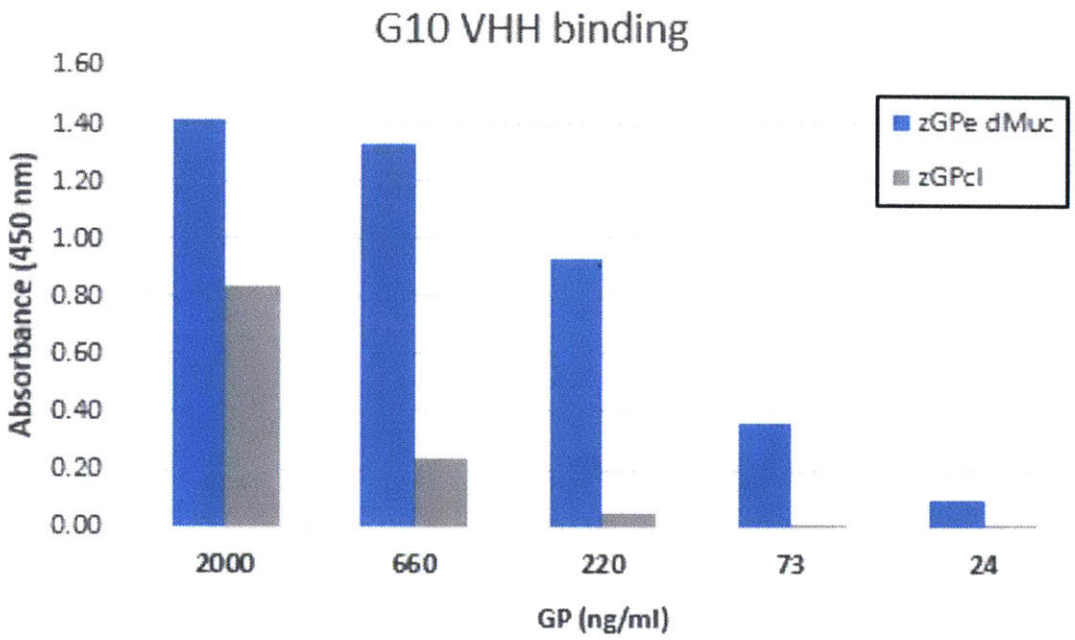
Supplemental 3: G10 and G84 pH sensitivity

ELISAs were performed on recombinant GP lacking the mucin domain (GPdM) in the respective pH conditions using biotinylated G10 or G84. ELISA were probed using secondary streptavidin: HRP and developed using TMB (Sigma). ELISAs were developed for 30 minutes at room temperature and quenched using 1 M HCl.



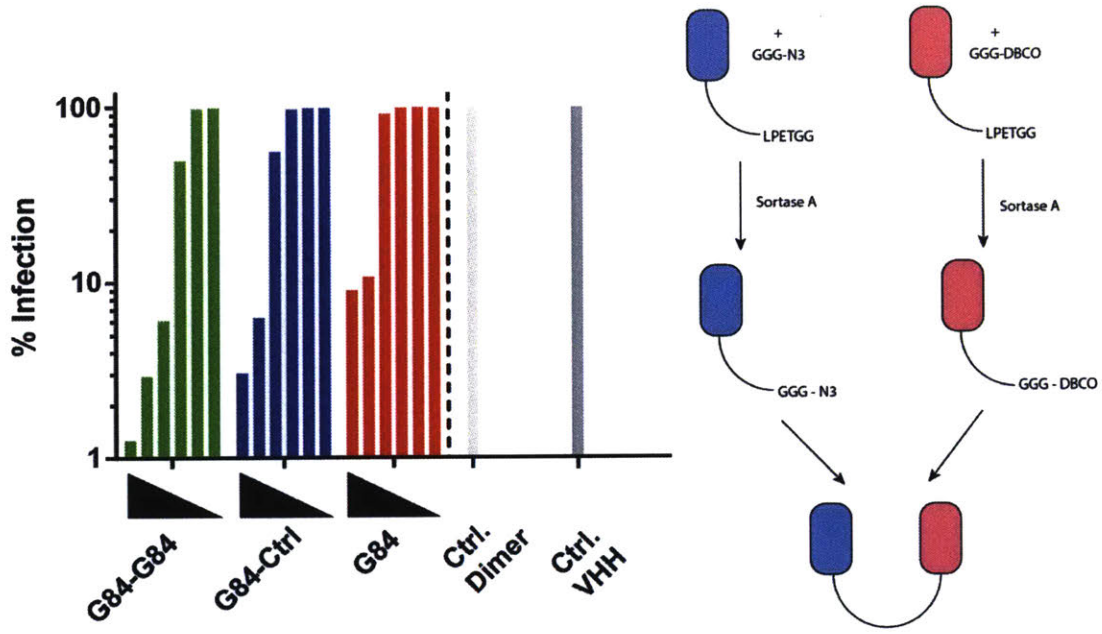
Supplemental 4: G10 does not cross-react with Bundibugyo Ebolavirus

Cross-reactivity of selected VHHs were tested against Zaire and Bundibugyo Ebolavirus glycoprotein. GP was also assessed in the requirement of the mucin like domain for binding. Work done be Crystal Moyer (Saphire Lab, TSRI)



Supplemental 5: Removal of the glycan cap via thermolysin perturbs G10 binding

G10 binding is assessed with differing concentration of either the mucin deletion or thermolysin cleaved GP to assess the binding for GP in these two states. Work done by Crystal Moyer (Saphire Lab, TSRI)



Supplemental 6: C-to-C VHH fusion or Ebolavirus neutralization

VHHs were either modified with a GGG-N_3 or GGG-DBCO at 5 times molar excess to VHH. Sortase was added to the reaction and incubated overnight at 4C. Sortase and unreacted VHH were removed from the reaction by addition of Ni-NTA. "Sortagged" VHHs were mixed at molar equivalents in order to facilitate N-to-N conjugation by click chemistry overnight at 4C. Unreacted VHHs were removed by spin filtration. Dimerized VHHs were subjected to EBOV neutralization to validate efficacy.

Chapter 3

Future Directions and Conclusions

In this thesis, I have described the identification of neutralizing VHHs against EBOV GP. Currently, I have not yet described the mechanism of neutralization of these VHHs. To our knowledge, these VHHs are the only that are capable of neutralizing infection of VSV-EBOV as well as live EBOV. These VHHs will be useful tools to probe the infectious cycle of EBOV or to understand GP interactions with host factors. Here I will outline future directions of this work as well as discuss the implication of these VHHs for the field of Ebolavirus research.

Future Directions

Multivalent and multifunctional VHHs

The use of sortase to append chemical handles to proteins has been well documented (Antos et al., 2017). Multivalent VHHs can be generated by combining different chemical handles to link VHHs of differing functions and specificities to achieve unique functionality.

Generation of multivalent VHHs through N-to-C fusions can create tandem VHHs that can recognize different epitopes simultaneously. This allows targeting of multiple sites on a protein antigen to increase avidity but also to modulate the neutralization profile or to increase the synergy of the interactions (D. J. Schmidt et al., 2016). By generating N-to-C fusions, *E. coli* can be used to produce them to high levels. One caveat is that it is possible that the N-to-C linkage might preclude binding if the N-terminus of the trailing VHHs are more sensitive to proximal residues. In this case, we can generate C-to-C fusion by modifying VHHs with a DBCO or azide functional handle to VHHs (Witte et al., 2013). Via click chemistry, VHHs can be attached by their C-terminus. We have demonstrated that this is possible with G84, where a C-to-C fusion of G84 increased neutralization of VSV-EBOV (**Supplemental 6**) While generation of

VHHs that are attached in tandem would prove to be useful, the need to examine competition of epitopes is needed to ensure that a bispecific VHH does not compete for the same epitope, effectively negating avidity effects.

Assessment of BL4 in vivo protection studies

Assessment of Ebolavirus neutralization has been addressed using cell-based infection assays. As antibodies that show promise in cell-based assays do not necessarily demonstrate protection, I intend to continue our congoing collaboration with UMRIID, we are already in talks to continue *in vivo* mouse infections experiments in order assess the validity of G10 and G84 as antivirals against Ebolavirus infection. For this, we will be using a mouse-adapted strain of Zaire Ebolavirus. The design of this experiment will be to determine if VHHs are protective against a lethal challenge of Ebolavirus.

Conclusion

The contribution of Fc in antiviral protection

The contribution of the Fc effector domain in *in vivo* viral protection is becoming more appreciated. Antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells can effectively mediate cell-mediated destruction of virus infected cells (Forthal, Landucci, & Daar, 2001). Human IgG unable to neutralize viral infection can recruit NK cells or other immune effector cells by FcγIIIa or FcγIIIb interaction, thereby conferring protection (Trotta et al., 2008). In a lethal influenza A (H1N1) challenge, rhesus macaques that were unable to produce neutralizing antibodies were able to keep an otherwise lethal challenge at bay (Jegaskanda,

Weinfurter, Friedrich, & Kent, 2013). Through the recruitment of immune effector cells through the Fc domain, non-neutralizing antibodies can be utilized for ADCC.

Mechanical neutralization of viral infection has been the prevailing hypothesis for our understanding of viral neutralization. Despite many antibodies being identified against EBOV GP, many were unable to neutralize *in vitro.* 168 identified Ebolavirus antibodies were assessed by their neutralization and protective profiles in order to determine whether the two are correlative. Their finding suggests that the effector function plays a much larger role than previously thought, where non-neutralizing antibodies can sometimes provide protection with a potent Fc effector function (Gunn et al., 2018). This observation adds an additional layer to our understanding of neutralizing antibodies, where previously non-neutralizing antibodies may be able to provide protection.

As VHHs do not contain an Fc domain, effector function is absent. Appending an Fc domain to a VHH has been achieved and can be performed recombinantly (Gunaydin, Yu, Graslund, Hammarstrom, & Marcotte, 2016). Thus, being able to swap in effector domains for a single VHH would be easier to achieve as the contribution of a light chain would not need to be considered. In addition, being able to express these VHH-Fc fusions in a mouse can be difficult, as a CH1 deletion is required as well as potential silencing of light chain expression. Realization of a mouse able to express VHHs with difference Fc will take time but can be achieved.

Continuing Viral Neutralization and Infection

VHHs G10 and G84 demonstrate the utility of antibodies as neutralizing agents. However, VHHs are not limited to the function ascribed to the epitope recognized. Many of the

VHHs identified in the Ebolavirus VHH library were not studied further due to their inability to neutralize virus. However, for many purposes the ability to perturb protein function is not needed and simply the need to recognize the target with high specificity is required.

For infectious diseases like Ebolavirus, many of the initial infections occur in areas that have limited access to the clinical laboratories and hospitals that would be able to triage and treat such infections. Paper diagnostics are emerging as point-of-care devices that would be able to diagnose an infection without the need of assays requiring special equipment. VHHs provide an added benefit for paper diagnostics that require recognition of a protein antigen, which the primary antigen is captured by a monoclonal antibody (Hu et al., 2014). VHHs are much more stable in higher temperatures and would be able to withstand the conditions needed in the field. VHHs would be able to complement if not replace monoclonal antibodies in these applications to bring paper diagnostics to fruition.

Targeting Ebolavirus Infection

The emergence of cell-based immunotherapies as a mode of targeting cancers using specific cellular markers expressed in cancerous cells has brought enormous success (Bucktrout, Bluestone, & Ramsdell, 2018). A similar approach can be achieved in infectious diseases. As many infections are resolved through the combined efforts of immune effector cells, it would be use to harness the specificity of VHHs to directly target infections in which the viral antigen is displayed on the surface, as many infected cells do. Expression of VHHs on T cells, or chimeric antigen receptor cells (CAR-T), can be used to target the tumor microenvironment (Xie et al., 2019). As such, display of VHHs the are specific to viral surface proteins allows for discerning of

infected and uninfected cells and eliminate viral factories from producing more virus. Use of cell-based immunotherapies allows for greater control of chronic infections that might be more difficult to target with the normal immune response without additional assistance.

Antibody drug conjugates can also provide a parallel approach in order to leverage VHHs in delivery. As cells only express GP when infected, it can be used as a cellular marker to differentiate between infected and uninfected cells. VHHs are easily modified with a sortase motif allow conjugation to drugs that can specifically target Ebolavirus infection. There is increasing usage of repurposed drugs such as nucleoside analogs to inhibit replication. These drugs have limited use due their lack of specificity, affecting cells that might not be infected. By Conjugating VHHs specific to a displayed viral antigen, targeted drug delivery for infectious disease can be achieved.

Informing Vaccine Design

Monoclonal antibodies have long been a mainstay for biotechnology and therapeutics. Targeting a specific antigens of interest that have therapeutic potential generates immense value over the lifetime of the product. Defining the epitope of these antibodies are incredibly important as this can illuminate how the antibody works and how this information can be leverage in order to develop more targeted and potent products.

Available vaccines for Ebolavirus infection is very limited; the goal being to eventually develop a vaccine or therapeutic that would be cross reactive amongst most, if not all strains of Ebolavirus. There is currently one Ebolavirus vaccine undergoing clinical trials, rVSVΔ-ZEBOV-GP, an EBOV vaccine composed of replication competent VSV that expressed EBOV GP. VSV is

harmless to healthy individuals, this vaccine has been quite successful in the use in ring vaccination field studies. In addition, there is another emerging vaccine derived from adenovirus and modified vaccinia virus (Q. Zhang & Seto, 2015; Feldmann, Feldmann, & Marzi, 2018; Sheridan, 2018; Suder, Furuyama, Feldmann, Marzi, & de Wit, 2018). There is also push for post-exposure prophylactic treatments against Ebolavirus, in which administration of VHHs may prove advantageous.

Other strategies for therapeutics are passive immunizations in which, historically, serum from protected individuals can be administered to infected individuals in order to confer protection, providing therapeutic value. Currently, monoclonal antibodies are being systematically identified to investigate the protective immune response. The next step in this process is using structure-guided vaccines in order to elicit the immune response towards epitopes of known function.

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