THE EBOLA VIRUS DELTA-PEPTIDES ARE ENTEROTOXIC VIROPORINS \textit{IN VIVO} AND POTENTIALLY DRUGGABLE TARGETS

AN ABSTRACT SUBMITTED ON THE TWENTY-FIFTH DAY OF MARCH TWO THOUSAND TWENTY TO THE DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE GRADUATE SCHOOL OF TULANE UNIVERSITY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY BY

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SHANTANU GUHA

APPROVED:

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WILLIAM C. WIMLEY, Ph.D. ADVISOR

_____________________________  ______________________________

DIANE BLAKE, Ph.D.  SAMUEL LANDRY, Ph.D.

_____________________________  ______________________________

HEE-WON PARK, Ph.D.  LISA MORICI, Ph.D.
ABSTRACT

During the 2013-2016 West African outbreak, severe gastrointestinal symptoms were common in Ebola patients and associated with poor outcome. The efficient spread of Ebola virus (EBOV) via vomitus and diarrheal fluids, which contain high concentrations of virus, likely contributed to the scale of the outbreak. The delta-peptide is a conserved product of post-translational processing of the abundant EBOV soluble glycoprotein (sGP). Here, the murine ligated ileal loop model, which is well-established for the study of diarrheal disease, was used to demonstrate that delta-peptide is a potent enterotoxin. Dramatic intestinal fluid accumulation peaked at 9-12 hours following injection of biologically relevant amounts of delta-peptide into ileal loops, along with gross destruction of the villous architecture, loss of goblet cell polysaccharides, and secretion of pro-inflammatory cytokines. Transcriptomic analyses showed that delta-peptide triggers immune and cell survival responses. Delta-peptide may contribute greatly to EBOV-induced gastrointestinal pathology in humans. These findings demonstrate that the EBOV delta-peptide is an enterotoxic viroporin and may be categorized as a novel virulence factor. We then hypothesized that the delta-peptide may also be a druggable target to explore a new avenue for novel EBOV therapeutics, which are direly needed. An unconventional coupling strategy was employed to conjugate a modified version of the 23-residue delta-peptide to a carrier protein Keyhole Limpet Hemocyanin (KLH) in order to immunize rabbits so that they may generate high-affinity binding antibodies against the delta-peptide. Antisera was collected from the rabbits after regular immunizations and the IgG fraction of the antisera demonstrated binding and recognition against several delta-peptide variants confirmed by Western blotting and ELISAs. We then used this knowledge to determine therapeutic index in vitro by testing the antibody against the delta-peptide in the context of synthetic PC-PG vesicles and CHO cells. The purified IgG was then tested against the peptide in vivo to determine therapeutic efficacy by returning to the mouse model of diarrheal pathology mentioned previously. In a small pilot experiment, we were able to successfully block the enterotoxic activity of the delta-peptide and did not observe gastrointestinal distress in the mice that were treated with
the peptide and antibody together versus just the peptide alone, signifying that the delta-peptide is a druggable target and may reveal a new therapeutic avenue against EBOV pathogenesis.
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HEE-WON PARK, Ph.D.                 LISA MORICI, Ph.D.
DEDICATION

I would like to dedicate this dissertation to Nicole, you are my sun and moon.
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I want to thank the city of New Orleans for opening my eyes and mind to life outside of Texas. I have learned so much from living here and I will carry New Orleans with me everywhere I go. And finally, I would like to thank all of the friends that Nicole and I have made during our stay in New Orleans and although there are too many to list, our experience would not be a fraction as fulfilling had we not met all of you. We have shared so many experiences and these are memories that we will cherish for our entire lives; I hope we were able to enrich your lives as much as all of you have enriched ours.
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CHAPTER 1: An introduction to the Ebola virus and the highly conserved gene product, the delta-peptide

1.1 The Ebola virus – a brief history and outlook for the future

The Ebola virus (EBOV) was first discovered to be pathogenic in humans during the 1976 outbreak in Sudan. In the decades that have passed since then, numerous outbreaks of EBOV have occurred globally with varying degrees of severity. However, in December 2013, one of the most devastating outbreaks of Ebola virus began in the West African country of Guinea. The peak of the outbreak was in late 2014 when more than 28,000 cases and 11,000 deaths were reported. Additionally, new outbreaks of Ebola continue to be reported into 2020 (Figure 1-1); currently, the Kivu outbreak in the Democratic Republic of Congo (DRC) is being addressed on a global scale as roughly 3,800 people have been infected and 2,200 have died; the World Health Organization (WHO) declared the outbreak a Public Health Emergency of International Concern (PHEIC) in July 2019. These more recent outbreaks also include cases of pathology which show unique immune evasion tactics by the virus. By hiding in immune-privileged compartments such as the testes, eyes, mammary glands, and cerebrospinal fluid (CSF), these viral strains can persist long term and contribute to higher morbidity and mortality. In addition to this, there is massive civil unrest and there are several war zones in the DRC; there is a general distrust for foreign public health workers and response teams, who a large portion of the population views as part of the “state government,” which makes outbreak intervention even more challenging. Groups such as Doctors without Borders/Médecins Sans Frontières (MSF) have even gone to lengths such as asking for military backup when setting up mobile healthcare units since many such teams have been met with violence and numerous public health workers have been killed.

There are numerous previous and ongoing vaccine studies testing efficacy, coverage, and safety. Many have passed from animal model to clinical trial, currently, only the ZEBOV-modified
VSV/Adenovirus platform vaccine has reached Phase 4 clinical trials in humans and has shown protection in extensive studies with non-human primates and humans\textsuperscript{19–25}. The VSV-ZEBOV vaccine has been prequalified by the WHO to combat the Kivu outbreak in the DRC, however there is an overwhelming shortage of vaccines when compared to the national population\textsuperscript{26}. Treatment of EBOV has proven to be a challenging endeavor as well. Many available therapeutics treat only the symptoms of Ebola virus pathology but are not antiviral drugs. Antivirals that were specifically designed for other viruses show varying degrees of success against EBOV\textsuperscript{27–30}. More recently, a robust and concerted effort has been made to develop specific strategies for stopping EBOV pathogenesis\textsuperscript{31–34}. These strategies include development of antibody cocktails which are specific for various viral components, and some of these have shown promise\textsuperscript{35}. For the most part, there are no especially efficacious treatment options for patients experiencing EBOV pathogenesis 2-3 days after initial infection\textsuperscript{36}. The average case fatality rate of EBOV is approximately 50%, with case fatality rates ranging from 25% to 90% in past outbreaks\textsuperscript{1–6,8,11,37}. There is a dire need for efficacious therapeutics against EBOV post-infection as outbreaks are regularly occurring.

EBOV has multiple virulence factors that can lead to the presentation of an array of different symptoms in patients\textsuperscript{8,38–41}. Some virulence factors of EBOV have been identified and extensively characterized, such as VP35 and glycoprotein (GP)\textsuperscript{39,42–44}. Interestingly, EBOV surface glycoprotein expressional levels also seem to correlate to virus production and infectivity\textsuperscript{39,45}. Additionally, in the 2014 outbreak, there were many statistically significant differences in case presentation and symptomology between fatal and nonfatal Ebolavirus disease (EVD), which include weakness, dizziness, and diarrhea\textsuperscript{8}.

1.2 Glycoprotein editing and subsequent production of the delta-peptide

The EBOV glycoprotein gene can produce multiple variants of the protein that it encodes\textsuperscript{46,47} (Figure 1-2). EBOV uses its spike glycoprotein (GP) to bind several human cellular factors and
receptors (TIM-1, scramblases, etc.) to gain entry and infect the cell via micropinocytosis and endosomal uptake\textsuperscript{48–52}. The full-length glycoprotein (GP1/2), which is embedded in the viral membrane comprises 10-24\% of the total gene products made from the GP gene and is a trimer. Small soluble glycoprotein (ssGP) is produced in small amounts (<2\%) and is a dimer. Interestingly, soluble glycoprotein (sGP) is produced as a dimer in large amounts (75-90\%) and is the majority gene product made with respect to the total GP gene products. The EBOV delta-peptide is derived from a signalase furin-mediated cleavage of the soluble glycoprotein (sGP) (Figure 1-2), which is a previously established phenomenon and has previously been implicated in increasing the pathogenicity of EBOV\textsuperscript{53–55}. It is quite likely that both sGP and the delta-peptide are produced by infected cells in appreciable quantities, though many details on exactly how these factors are secreted are still unclear. Several filoviruses including Ebola virus, Cuevavirus, and Reston virus all have encoded and conserved delta-peptides\textsuperscript{46,56}. Previously, structural modeling revealed similarity between the delta-peptide of EBOV and NSP4 of rotavirus, a known enterotoxic viroporin\textsuperscript{57,58}. A viroporin is a virally-derived peptide or protein that contributes to the pathogenicity or propagation of the virus\textsuperscript{59,60}. Viroporins may also activate the NLRP3 inflammasome in some cases\textsuperscript{61}. Recently, compelling and robust data has been generated by He et al showing that the delta-peptide in its oxidized form has potent permeabilizing viroporin activity in numerous cell types in vitro\textsuperscript{62}. This was further explored by Pokhrel et al, who showed Molecular Dynamics (MD) simulations in which they described the influx of certain cations after the peptides formed into multimeric ion channels within a simulated membrane\textsuperscript{63}.

Viroporins, contribute to the pathogenicity or propagation of the virus that encodes them, sometimes through perturbation of the host cell membrane to aid in the release of newly formed virions\textsuperscript{59}. The high level of conservation of the delta-peptide, especially across numerous EBOV variants, informs of the importance of the peptide to the viral genome\textsuperscript{57,62}. However, the EBOV delta-peptide seems to have an internal on-off switch for activity as well. The sequence of the delta-
peptide reveals two conserved cysteines at the C-terminal region. When the cysteines are oxidized to a cystine to form a hairpin, the peptide has strong pore-forming potency; however, when the cystine is reduced, the peptide loses its pore-forming ability by orders of magnitude. The purpose of this on-off switch is yet to be determined, but it is expected to give the delta-peptide more activity in the oxidizing extracellular milieu than in the reducing environment of the cell cytosol. The concentration of circulating sGP and delta-peptide in serum has not been reported for patients which are acutely infected with EBOV, although it is reported that shedding occurs early and in large quantities\textsuperscript{47,64,65}. However, \textit{in vitro}, it has been reported that eukaryotic cells which are infected with live EBOV produce sGP at a concentration of 2 µg/µL and delta-peptide at a concentration of 0.5-1 µg/µL\textsuperscript{66}.

EBOV delta-peptides are 40-49 residues depending on the Ebola subtype, however they may be cleaved to smaller species by serum exopeptidases as they circulate through the vascular system of an infected individual\textsuperscript{46}. The 23-31 residues on the C-terminal portion of the delta-peptide are more stable in the presence of proteases found in serum\textsuperscript{46}. Hereinafter, EBOV delta-peptide nomenclature will be used regularly (E23ox refers to EBOV delta-peptide, 23 C-terminal residues, oxidized). All delta-peptide sequences used in this study were derived from the Zaire EBOV genome.

Interestingly, survivor serum samples from previously infected patients have shown antibody reactivity to the delta-peptide by ELISA testing (Figure 1-3). This data suggest that the delta-peptide is made in large enough quantities that the survivors mounted an antibody response against it and having antibodies reactive to the delta-peptide may potentially aid in survival. This information, coupled with previous \textit{in vitro} studies which show exertion of pore-forming activity justified further exploration of the overall relevancy of the peptide to the pathogenicity and propagation of the virus\textsuperscript{62}. 
Virulence factors in Ebola pathogenesis can be traced to certain genes in the Ebola genome\textsuperscript{38}. In this dissertation, we propose that the EBOV delta-peptides are a small, previously uncharacterized family of EBOV virulence factors that can contribute to gastrointestinal distress via diarrheal syndrome. This may increase pathogenicity and potentially the transmissibility of EBOV during active infection of the patient. EBOV delta-peptides are viroporins which are also able to exert severe enterotoxic effects on the small intestines.

**1.3 A potential role for the delta-peptide during active infection**

The EBOV delta-peptide has been studied previously and has been proposed to be a functional portion of EBOV pathogenesis, but functional studies of peptide activity alone are sparse. The delta-peptide was first postulated to be a non-structural expression gene product of the GP gene in 1999\textsuperscript{54} (Figure 1-4A). In 2005, it was determined that the delta-peptide is produced in a monomeric state and does not induce the production of pro-inflammatory cytokines at a concentration of 50 µg/mL \textit{in vitro}\textsuperscript{66}. It was further determined that the delta-peptide is not responsible for endothelial cell activation, nor does it affect endothelial cell barrier function\textsuperscript{67}. However, in 2011, it was discovered that Fc-tagged delta-peptides (immunoadhesins) were able to inhibit the infective capacity of certain subtypes of EBOV, suggesting that the delta-peptides have an active role in infection of cells and subsequent pathogenesis\textsuperscript{56}. In 2015, the delta-peptide was modeled against NSP4 of Rotavirus and was proposed to have a potentially lytic peptide sequence\textsuperscript{57}. In 2017, it was characterized as a functional viroporin in cell culture as it was found to permeabilize numerous eukaryotic cell types, cause loss of synthetic bilayer resistance, and allowed for the release of small molecules from synthetic vesicles\textsuperscript{46}. The sequences of delta-peptides from several filoviruses including five from the EBOV family are shown in Figure 1-4B\textsuperscript{46}. Survivor serum from patients in the most recent outbreak appear to contain antibodies that react positively to the delta-peptide (L. Melnik and RF Garry, unpublished, Figure 1-3). In 2019, the EBOV delta-peptide was predicted by molecular dynamics simulations to partition into membranes, forming pentameric and
hexameric ion channels, which allow the flux of certain ions. In a matter of 20 years, only these few findings have been reported regarding the delta-peptide. These previous findings over the course of two decades have led to the studies described in this dissertation.

1.4 Mechanism of the delta-peptide viroporin activity

Initially, the delta-peptide was proposed as a peptide ion channel by Gallaher et al, due to structural similarity to NSP4 of Rotavirus. Further examination of the peptide by He et al showed that the delta-peptide can allow for fractional loss of resistance in a surface-supported lipid bilayer reporter system. Furthermore, ion dysregulation is considered to be a hallmark of diarrheal syndrome.

We tested this potential phenomenon in vitro using CHO cells to provide further details to the mechanism of action for the delta-peptides. Ionophores, which are ion-indicating fluorescent dyes, can fluoresce in the presence of very specific ions and were loaded into the cells and treated with different variants of the delta-peptide. To track influx of Ca²⁺, Indo-1 AM was used and to track influx of Na⁺, Sodium Green was used. Both ions have an extracellular concentration that is higher than the intracellular concentration and would likely enter the cell to equilibrate the membrane potential. Within 1 hour of treatment with the delta-peptides, the cells increased in fluorescence compared to the untreated controls when testing for both Ca²⁺ and Na⁺ (Figure 1-5A). E23ox and E40ox have been mentioned previously in this study. MelP5 is a well-categorized pore-forming peptide, E23A is a variant of E23 where the cysteines of the C-terminal hairpin are mutated to alanines (acting as a surrogate for the reduced form of E23ox), and E18n refers to first 18 residues of the N-terminal portion of E40. In Figure 1-5B, this effect may be visualized over a period of 120 minutes after administration of 100 μM E23ox to the CHO cells and the Indo-1 becomes fluorescent as Ca²⁺ fluxes into the cell through potential E23ox ion channels. In Figure 1-5C, using confocal microscopy, the ion flux may be specifically visualized using a dye-labeled variant of E23ox using AF555 (red), a 10k dextran-AF488 to delineate intact cellular membranes (green), and Indo-1, AM to indicate Ca²⁺ ion flux (blue). The colocalization of the three channels in the merged image
indicate that where the dye-labeled peptide was concentrated, there was also ion flux and dextran entry. Not all cells were affected, but often, peptide permeabilization of cellular membranes functions in a stochastic fashion. A z-stack of this effect can be seen in Figure 1-5D, where the dye-labeled peptide is seen accumulating on the cell membrane, but also along the nuclear envelope, which is a novel observed feature of the EBOV delta-peptide. When comparing an indiscriminate pore-former like MelP5 to E23ox, even at low concentrations, the same effect is not achieved (Figure 1-5A). Only the cells treated with E23ox are able to fluoresce. The cells treated with MelP5 are not because MelP5 forms large pores that allow the ion sensitive dyes to escape from the cells. When a hybrid experiment was conducted to monitor Ca$^{2+}$ flux and SYTOX Green entry simultaneously, it appeared that ion conductance occurs at a faster rate initially than SYTOX Green entry. (Figure 1-5E) Overall, this series of experiments indicate a potential mechanism of action for the delta-peptides, which is in accordance with past experiments found in the literature, as well as the data produced in this study. The delta-peptides may induce gastrointestinal distress via diarrheal syndrome by way of partitioning into cellular membranes and causing severe ion dysregulation.

### 1.5 Effects of the delta-peptide on synthetic vesicles and eukaryotic cells

He et al. demonstrated the effects of the delta-peptide on synthetic vesicles and eukaryotic cells. The authors found that the delta-peptides in their oxidized form (with internal cystines) were able to permeabilize synthetic vesicles and allow the release of small molecules (Figure 1-6A). Additionally, eukaryotic cells (HeLa, CHO, Vero, and MDCK) treated with oxidized delta-peptides allowed a membrane-impermeant nucleic acid-intercalating dye SYTOX Green to cross the membrane, bind to DNA, and fluoresce after one hour of treatment (Figure 1-6B). This data can be further supplemented by observing fractional SYTOX Green entry into human umbilical vein endothelial cells (HUVECs), which may have further implications into the characterization of the delta-peptides in vivo (Figure 1-6C). When testing the delta-peptides in combination with each
other (ie. E23ox + E40ox) and keeping the total peptide content consistent, the delta-peptides do not exert activity in a synergistic way (Figure 1-6D). This may signify that many delta-peptide variants, which are likely present and systemically circulating in a patient experiencing acute EVD are able to elicit the same effects as a singular delta-peptide variant alone, further exemplifying that multiple delta-peptides of varying amino acid length are able to permeabilize eukaryotic cells in a potentially modular fashion.

1.6 Goals of the research presented in this study

The information in the preceding sections led us to hypothesize the delta-peptides’ role in EVD pathogenesis. One of the primary goals of this study is to characterize the effects of this peptide in the murine small intestine to determine the purpose of the EBOV delta-peptides and potentially explain the conserved nature of this peptide within many EBOV subtypes (Zaire, Sudan, Bundibugyo, and Tai Forest) as well as other members of the Filoviridae family.

Aim 1: Testing the hypothesis

We hypothesized that the conserved EBOV delta-peptides can induce diarrheal syndrome, potentially affecting EVD pathology. Our initial research aim was to study the behavior of several EBOV delta-peptides of varying amino acid length in a closed environment in vivo, to control for confounding factors, but to maintain relevancy to the overall progression of EVD pathology. As such, a relevant murine model of diarrheal pathology was chosen where a surgical ligation of the small intestine is performed to create a ‘closed’ ileal loop. The ileal loop is then injected with a syringe to introduce the delta-peptide at relevant physiological concentrations into the lumen of the small intestine within the loop. This closed ileal loop can then be individually monitored through necropsies at pre-determined time points rather than looking at gross pathology of the whole mouse with systemic delta-peptide treatment. This method also allows for a way to monitor the action of the delta-peptide without being cavalier in consideration to the mice which undergo this surgery
and without being wasteful to laboratory resources. Several of the EBOV delta-peptide variants (E17ox, E23ox, and E40ox) induced diarrheal pathology in the closed ileal loops, while peak activity appeared 9 to 12 hours after introduction to the loop. We used histological analysis to confirm the pathological effects of the delta-peptides and were able to determine that the EBOV delta-peptides exert potent enterotoxic effects on the small intestine.

**Aim 2: Engineering a delta-peptide antigen**

Having established the delta-peptides as functional enterotoxic viroporins, we sought to engineer an antigen in order to produce high-affinity neutralizing antibodies against the delta-peptide, specifically E23ox. We designed a novel strategy to conjugate a modified version of the delta-peptide to a carrier protein called Keyhole Limpet Hemocyanin (KLH), which is an industry standard in antigen conjugation for custom specific antibodies. This antigen was then used to immunize two rabbits on a regimented boosting schedule for several months and production bleeds were collected at regular intervals to obtain the antisera containing the specific antibodies produced against the delta-peptide. This approach resulted in two rabbits that produced polyclonal antibodies capable of binding several delta-peptides of varying amino acid length.

**Aim 3: Characterization of anti-delta-peptide antibodies**

Having engineered an antigen which was capable of producing specific antibodies against the delta-peptide, we sought to investigate numerous affinity purification strategies that would result in pure specific IgG samples. We then further tested the applicability of the anti-delta-peptide antibodies by pursuing avenues such as the preclinical characterization of therapeutic index and potential as an EBOV diagnostic tool, as current diagnostic techniques rely on PCR. Using a combination of synthetic vesicles and eukaryotic cells to monitor blocking of the delta-peptide viroporin activity, we were able to determine that the antibody is also able to block diarrheal pathology induced by the delta-peptides when returning to the aforementioned murine diarrheal pathology model. Our
results indicate that the EBOV delta-peptides are enterotoxic viroporins and may also represent a novel target in EBOV therapeutic design. We ultimately propose to test the antibody as part of an antibody cocktail in a non-human primate (NHP) model of live EBOV infection in conjunction with collaborating labs which are equipped to conduct such experiments in order to evaluate therapeutic potential. We also propose to develop a novel diagnostic test for EVD based on the early circulating production of delta-peptide by the virus.
Figure 1-1: EBOV outbreaks by size and species since 1976

This map published by the CDC on their website provides a historical and geographic distribution of EBOV outbreaks. The Zaire, Sudan, and Bundibugyo strains of EBOV are responsible for the largest outbreaks in Africa; the Zaire strain has the highest case fatality rate. It was associated with the largest outbreak on record from 2014-2016 which resulted in 28,600 cases and over 13,000 deaths. It is also associated with the ongoing outbreak in the DRC. This image is attributed to the
CDC in their online resource of Ebola viruses (https://www.cdc.gov/vhf/ebola/history/distribution-map.html)\textsuperscript{71}. 
Figure 1-2: Glycoprotein splicing and variant gene production

The glycoprotein gene produces multiple versions of the protein due to differential RNA splicing and editing at a conserved site containing 7 uridines. The addition of one non-templated uridine caused by RNA slipping produces full-length glycoprotein (GP), which is converted to GP1/2 by cleavage at a signalase furin site and becomes the transmembrane protein of the virion membrane as a trimer. Full-length GP comprises 10-24% of the total gene products made by the GP gene. The addition of two non-templated uridines produces small soluble glycoprotein (ssGP), which comprises <2% of the total gene products made by the GP gene and is produced as a dimer. The unedited RNA transcript produces soluble glycoprotein (sGP) and comprises 75-90% of the total gene products made by the GP gene and is also produced as a dimer. However, the delta-peptide is produced at this stage via cleavage by an alternate signalase furin site of sGP, producing a 40-residue C-terminal peptide. It is likely that delta-peptide and sGP are produced in relatively
equivalent amounts since the delta-peptide is a cleavage product of sGP\textsuperscript{46.54}. This figure is attributed to Dr. Robert F. Garry, our collaborator on this project.
Recent unpublished data shows the immune reactivity to the EBOV delta-peptide by testing the serum from survivors collected from patients during the 2014-2016 outbreak. This data seems to suggest two things: 1) the delta-peptide is made in large enough quantities that the survivors mounted an antibody response against it and 2) having antibodies reactive to the delta-peptide may potentially aid in patient recovery from acute EVD pathogenesis. The EBOV delta-peptide may potentially be a novel immunologic target for therapeutic development. This figure is attributed to the lab of Dr. Robert F. Garry, a collaborating lab on this project.
Figure 1-4: First description and sequences/structures of the EBOV delta-peptides

(A) A schematic representation of the EBOV sGP and subsequent processing to produce delta-peptide, first described in 1999. This figure is attributed to Volchkova. (B) A comparison of delta-
peptide sequences encompassing four EBOV subtypes (Zaire, Sudan, Bundibugyo, and Tai Forest) which are pathogenic to humans of the Filoviridae family, the Lloviu cuevavirus of the Filoviridae family, and Reston subtype of EBOV which is pathogenic in non-human primates. This figure is attributed to He at al\textsuperscript{46}. 
Figure 1-5

A

Indo,1 AM Fluorescence

Sodium Green Fluorescence
Figure 1-5: The EBOV delta-peptides act as nonspecific ion channels *in vitro*

(A) When using the ionophores Indo-1 AM and Sodium Green, which indicate Ca\(^{2+}\) and Na\(^+\) respectively, it is possible to observe an increase in fluorescence indicative of specific ions moving into the cell cytosol and the ionophores fluoresce in response. Both plots are background subtracted using the untreated line as the baseline. E23ox and E40ox are able to allow passage of Ca\(^{2+}\) ions intracellularly and E23ox is also able to allow passage of Na\(^+\) ions as well. E40ox is above background, but less effective for the transport of Na\(^+\) ions. (B) A visualization of Indo-1, AM fluorescence over time where the left panel represents no treatment and an image taken at 30 minutes after the start of the experiment, the middle panel represents E23ox treatment at 30 minutes after the start, and the right panel represents E23ox treatment 120 minutes after the start of the
experiment. (C) Confocal images which show E23ox doped with a dye-labeled variant of E23ox using AF555 to track membrane binding (red), a 10k dextran-AF488 to delineate intact cellular membranes (green), and Indo-1, AM to indicate Ca$^{2+}$ ion flux (blue). At 0 and 90 minutes, the untreated panel shows minimal activity and signal. However, for the E23ox-treated panel, it appears cell-binding has already started at the 0 minute time point, and by 90 minutes, the colocalization of the three channels in the merged image indicate that where the dye-labeled peptide was concentrated, there was also ion flux and dextran entry. (D) A z-stack from the confocal images taken for the previous section also shows that E23ox accumulates on the cell membranes and also can be found on the nuclear envelope. (E) A designer assay developed to track the fractional entry of SYTOX Green and the level of Ca$^{2+}$ flux by Indo,1 AM over the course of 60 minutes at 5 minute intervals (background not subtracted). Although MelP5, our pore-forming positive control peptide, allows high SYTOX entry, it does not allow Ca$^{2+}$ flux. E23ox and E40ox can be seen here allowing fractional SYTOX entry and Ca$^{2+}$ ion flux by Indo,1 AM at a concentration of 100 µM.
Figure 1-6

A
Figure 1-6: The effects of the EBOV delta-peptides on synthetic vesicles and numerous eukaryotic cells
(A) The effects of the EBOV delta-peptide on synthetic vesicles of different compositions (PC & PC/PG) which have a fluorophore and quencher entrapped. For PC vesicles, the oxidized delta-peptide variants are able to elicit an increase in fluorescence by permeabilizing the vesicles. For the PC/PG vesicles, they are permeabilized by both oxidized and reduced delta-peptide variants, which indicates a sensitive platform for measuring delta-peptide permeabilizing activity. This figure is attributed to He et al.46. (B) When testing the oxidized and reduced versions of the delta-peptides against nucleated eukaryotic cells (HeLa, Vero, CHO, and MDCK), fractional SYTOX Green entry can be quantified. In this platform, only the oxidized peptide variants are able to permeabilize the membranes to SYTOX Green entry. This figure is attributed to He et al.46. (C) When testing permeabilization against Human Umbilical Vein Endothelial Cells (HUVEC), a similar effect is seen where fractional SYTOX Green entry coincides with increasing concentrations of the EBOV delta-peptides. (D) When using the delta-peptides in combination against CHO cells, but keeping overall protein content the same, combinations treatments are also able to elicit similar effects as seen with singular peptides. This may signify that the delta-peptides work in a modular fashion, but this modularity does not entail synergism.

CHAPTER 2: The in vivo effects of the Ebola virus delta-peptide in the context of gastrointestinal distress

Introduction

Once the in vitro effects of the delta-peptide had been characterized, it was necessary to determine the physiological relevance of its biological activity. It is well-documented that EBOV can infect numerous cell types and organ systems41. The virus begins by targeting and dysregulating several types of immune cells, such as dendritic cells, which are canonically antigen-presenting cells and can coordinate T-cell responses among many other functions72–74. It also targets macrophages,
which are part of the innate immune system\textsuperscript{72–74}. The virus is able to replicate and use the vascular system to spread to many organs such as the liver, lungs, and gastrointestinal tract\textsuperscript{41}. As the virus spreads, numerous symptoms are elicited including fever, headache, weakness, diarrhea, pain, vomiting, conjunctivitis, and edema\textsuperscript{8}. There are several reports of severe gastrointestinal (GI) involvement in EVD progression, often this symptomology is indicative of fatal EVD versus nonfatal EVD\textsuperscript{8,75,76}. Since EBOV strains are classified as BSL-4 level & Category A biothreat agents by the United States, we are restricted to using non-replicating viral subunits such as the delta-peptide\textsuperscript{77}. However, in this study, we were able to elucidate the role of delta-peptide for this reason as the results were not confounded by any other effects elicited by other viral proteins. A GI-targeted approach was taken to address the potential role of the delta-peptide in the GI distress associated with EVD.

Diarrheal pathology is noted early in EVD progression and offers a system which can be visually monitored for multiple symptoms. A murine model of diarrheal pathology, developed using \textit{V. cholerae}, was selected for this study since the induced pathology can be visualized in ligated 2 cm sections within the small intestine\textsuperscript{78}. Several EBOV delta-peptides were tested for effects on the small intestine environment, including E15ox, E17ox, E23ox, E40ox, and E23scr (a scramble variant of E23ox). The sequences of all delta-peptide variants used in this study can be found in Figure 2-1. In the model, the small intestine is surgically ligated just below the caecum and the luminal space is injected by a high-gauge syringe to introduce the delta-peptides as the experimental group, 0.025\% acetic acid (AcOH) as the vehicle of the peptide for the negative control group, and a sublethal dose of 1 x 10\textsuperscript{7} CFU \textit{V. cholerae} as indicated by the original paper for the positive control group. To validate the model, we used cholera toxin (CTX) to ensure that diarrheal pathology can be measured using our metric, termed Loop Ratio (loop weight (g)/loop length (cm)). Mice are also considered to be a model platform for studying acute intestinal inflammation; as such, BALB/C mice were selected for this study\textsuperscript{79}. After a pre-determined time-
point had been reached post-ligation and injection, each mouse underwent a necropsy in which the ligated ileal loop was resected from the mouse and then weighed and measured before fixing the tissue in EtOH/paraformaldehyde. The fixed tissue was then embedded in paraffin and intestinal cross-sections were cut to observe histopathology. Immunohistochemistry and immunofluorescence were used to observe the architectural changes associated with delta-peptide treatment. Additional tissue samples were saved and processed to extract the RNA to observe the transcriptomic-level effects elicited by E23ox. Thus, we are able to present a comprehensive in vivo characterization of individual EBOV delta-peptides in the context of the small intestine and gastrointestinal pathology.

Materials and Methods

Preparation of peptide

Each peptide used in this study was synthesized by Bio-Synthesis Inc. (Lewisville, TX) and was delivered to us at >95% purity as confirmed by HPLC. To oxidize the C-terminal cysteines into an internal disulfide linkage, 20% DMSO (v/v) was added to peptide in 0.025% AcOH as an oxidizing agent overnight. The peptide solution was then dried under the vacuum to evaporate the excess DMSO. The peptide was then re-dissolved in 0.025% AcOH and compared to a reduced sample of peptide on HPLC. The disulfide linkage slightly increased the hydrophobicity and shifted the retention time peak of the peptide by ~10 seconds on an HPLC method designed for EBOV delta-peptide reaction tracking. Peptide cysteine alkylation was done by reacting reduced E23 (eluted in 0.025% AcOH) with iodoacetamide eluted in dimethylformamide (DMF) in molar excess to alkylate the sulfhydryl groups on the two cysteines in the sequence. The product was purified by HPLC fraction collection, confirmed on MALDI-MS, dried in the vacuum, and resuspended in 0.025% AcOH before used in the murine model described below.

In vitro – initial testing (SYTOX Green and ionophores Indo, 1 AM/Sodium Green)
Cells of different lineages (human HeLa, A549, HuVEC, canine MDCK, and rodent CHO) were seeded into 96-well tissue culture plates and grown to at least 90% confluence. The growth media (DMEM containing phenol red and 10% FBS) was exchanged for serum-free DMEM without phenol red during the assays. Peptides are serially diluted from a starting concentration of 50 to 100 µM in medium containing SYTOX Green. SYTOX Green is a small DNA-binding dye that is impermeant to cell membranes; when the membrane is permeabilized, the dye can enter the cell and fluoresces when complexed with nucleic acid. Each of these assays take kinetic reads for 1 hour at five-minute intervals at 37°C after introduction of the peptide to the cells. The SYTOX fluorescence intensity at 60 minutes was used to calculate fractional permeabilization of the plasma membrane as indicated by the positive control, a potent membrane-permeabilizing peptide called MelP5. When using the ionophores Indo, 1 AM and Sodium Green to track Ca^{2+} and Na^{+} flux respectively, the ionophore is prepared in DMSO and diluted 1:1000 in DMEM (without phenol red or FBS) and loaded into the cells in a 96-well plate for 1 hour to allow for the ionophore to cross the cell membranes. The ionophore-containing media is aspirated, and the cells are washed before adding either peptide alone diluted in media or peptide diluted in media with SYTOX Green as mentioned in Chapter 1 (Figure 1-5E). The plate reader is then set for the appropriate excitation and emission wavelengths, and a kinetic read for one hour is done.

**Mouse model – testing diarrheal pathology and gastrointestinal distress**

After our *in vitro* experiments indicated that the EBOV delta-peptide was able to exert potent permeabilizing effects on numerous cell types, we chose an established *in vivo* model of diarrheal pathology in mice. Originally, this model was used to visualize the effects of *Vibrio cholera* on the small intestine. In the model, each BALB/C mouse undergoes a surgery in which a 2 cm section just before the caecum is tied off with suturing silk to create a ligated ileal loop. The loop is then injected with a 100 µL volume of EBOV delta-peptide at varying concentrations ranging from 10
µM to 200 µM, sub-lethal concentrations of *V. cholerae* for a positive control, or 0.025% AcOH for a negative control (Figure 2-2A).

The mouse is then sacrificed and necropsied at pre-determined timepoints (6, 9, 12, and 24 hrs) to observe the effects on the ileal loop (Figure 2-2B). The primary metric that is used is the Loop Ratio (loop weight (g)/loop length(cm)) to determine fluid accumulation (Figure 2-2B). A normal loop ratio ranges from values between 0.03-0.04, an elevated loop ratio in which fluid is accumulating ranges from 0.05-0.06, and a diarrheal loop ratio is designated by us when the loop ratio is double the normal loop ratio or anything greater than 0.07+.

This model allows us to observe multiple aspects of activity of the EBOV delta-peptide: observing where the peak activity of the delta-peptide occurs after introduction into the ileal loop, concentration dependence on peptide activity to generate a diarrheal syndrome, activity of discrete delta-peptides of varying amino acid length, etc.

**Histology - PAS staining**

After each loop was measured and weighed, the whole loops were then imaged at a gross anatomic level. Following this, the loop was fixed and subsequently embedded in paraffin to conduct histological studies. Transverse cross-sections of the mouse small intestinal tissue were then stained using a Periodic Acid Schiff kit in order to detect polysaccharides and mucosubstances, which serve as an indicator for the presence of mucin-producing Goblet cells found along villous protrusions. Goblet cells produce mucin and release the mucosubstances into the lumen of the small intestine as an innate immune response to inflammation. Density of mucin-producing Goblet cells in small intestine cross-sections may thus indicate the level of inflammation caused by the EBOV delta-peptide. The counterstain is hematoxylin, which also allows us to score the architecture of the small intestine as well, giving us a multipartite approach to assessing damage exerted by the delta-peptide.
**Histology - Fluorescence staining (goblet + cytokines)**

As a complement to the PAS staining, additional fluorescent staining was done to further highlight the major architectural changes in the small intestine after introduction of the EBOV delta-peptide. Small intestine cross-sections were fluorescently stained with wheat germ agglutinin (WGA-AF488), a lectin which binds sialic acid and N-acetylglucosaminyl residues and DAPI as a nuclear counterstain. In some cases, TAMRA-phalloidin was also used to stain actin filaments, though this method was not used often. The WGA-AF488 allows for the staining of mucin-producing Goblet cells, as they contain many N-acetylglucosaminyl residues in their mucosubstances, and they also fluorescently label Paneth cells, found near the crypts of the villi in the small intestine.

**RNASEq**

During the course of the mouse experiments for the *in vivo* model of diarrheal pathology, ileal loop tissue from the expanded loops were resected and saved for RNA extraction. From the time of peptide introduction into the closed ileal loop to the time of necropsy, dramatic changes in tissue architecture occur. This likely results in the upregulation and downregulation of damage and repair genes at a cellular level in response to the inflammation and diarrheal syndrome. Since genetic changes usually occur early in the damage response timeline, RNA was extracted from several loops from the 6- and 9-hour time points for vehicle controls and peptide-treated animals. The changes in the transcriptome can yield valuable information and provide insight into the mechanism of action for the peptide as well as the inherent cellular responses to the delta-peptide. As such, the transcriptome between loops treated with E23ox will be compared to those treated with the negative control (0.025% AcOH, the eluent of the peptide) with an appropriately selected reference genome. Additionally, Gene Set Enrichment Analysis (GSEA) can be used to inform on several gene families using advanced gene clustering.

**Results**
Establishment of the in vivo model of testing diarrheal pathology

Gastrointestinal distress via diarrheal syndrome has previously been recorded as a symptom of EBOV pathogenesis and is a determinant in the lethality of that specific infection\(^8\). There is a previously established in vivo model of diarrheal pathology designed to look at the acute diarrheal effects of the enterotoxic bacteria *V. cholerae* and its major bacterial virulence product, cholera toxin, in the small intestine\(^7\)\(^8\). In short, the mouse is anesthetized using ketamine/xylazine and undergoes a surgery in which a midline incision is made, and the intestines are exposed. Once the caecum is located, a 2 cm section of the small intestine is ligated by using surgical thread to tie off both ends of the intestine, making a closed ileal loop. The closed ileal loop is then carefully injected with a high gauge syringe needle to introduce the delta-peptide. This procedure can be visualized in Figure 2-2A. Only 100 µL of volume may be injected as the volumetric space inside a 2 cm closed ileal loop is limited. After injection of the loop is complete, the mouse is then sutured and allowed to recover.

A time scale for necropsy and ileal loop resection was then developed to chart the action of the experimental compound, which can be seen in Figure 2-2B. The earliest time point was 6 hours, then 9 hours, then 12 hours, and finally 24 hours. Once the necropsy is completed, the ileal loop is weighed, and the length of the loop is measured. These measurements are used to determine fluid accumulation by Loop Ratios (the weight of the loop (g) divided by the length of the loop (cm)). The negative control for this portion of this study was the vehicle of the EBOV delta-peptide (0.025% AcOH) and average Loop Ratios for this group at all time points yielded ratios of 0.03 to 0.04, which we determined to be the baseline Loop Ratio (Figure 2-2B). A doubling of the baseline Loop Ratio indicates severe fluid accumulation and diarrheal syndrome. In order to establish the model as a viable tool in our study, purified cholera toxin (CTX) was tested as one of the positive controls to first validate the model as it is a very well-established enterotoxin. Even at a concentration of 1 µg/ileal loop, cholera toxin induced heavy fluid accumulation and diarrheal
syndrome as seen in Figure 2-2C. Using purified CTX, the model was validated for modified usage with the EBOV delta-peptide and an enterotoxic pathogen such as *V. cholerae*.

The EBOV delta-peptide was injected into the loop at a concentration of 100 µM and a positive control was also selected, the bacteria *V. cholerae* at a sublethal concentration of 1x10^7 colony forming units (CFUs) as indicated by the paper which describes the model. Loop Ratios for these experimental groups were charted over 24 hours (Figure 2-3A). Here, t-tests were used for statistical analysis. The EBOV delta-peptide used here was E23ox and was able to outperform *V. cholerae* at all time points monitored, with the peak activity at the 9- and 12-hour time points as seen in Figure 2-3B. Statistics here were calculated using a modified chi-square test. During the 9- and 12-hour time points the Loop Ratio for the mice that received E23ox more than doubled the baseline Loop Ratio, indicating severe fluid accumulation and diarrheal syndrome. This was also the first indication that EBOV delta-peptides can induce severe gastrointestinal pathology. The level of volume accumulation in the closed ileal loops may be observed in Figure 2-3C. Additionally, the mice that received the E23ox also exhibited signs of distress including hunching and grimacing.

**E23ox acts in a dose-dependent manner**

In the previous section, E23ox was demonstrated to induce gastrointestinal distress by way of diarrheal syndrome. The peptide was tested at several concentrations (lower than 100 µM) in order to determine whether it is possible to modulate the level of fluid accumulation (Figure 2-4A). Here, t-tests were used to determine statistical significance. Similar to the previous section, the peptide was injected into the ligated ileal loops of mice and the same time scale was used for ileal loop resection. When considering the 9- and 12-hour time points together as the peak of activity, concentrations used in this section were 10 µM, 20 µM, 50, 100, and 200 µM E23ox (Figure 2-4B). Here, an ANOVA test with multiple comparisons was conducted to determine statistical
significance. The 20 µM, 50 µM, 100 µM, and 200 µM concentrations caused severe fluid accumulation, especially at the time points for peak activity. Here, a stepwise increase in loop ratio occurs with increasing delta-peptide concentration. Although the 10 µM concentration did not yield significance in the statistical test, we can visualize the effect of the peptide nonetheless (Figure 2-4C). It is possible to modulate the level of fluid accumulation via peptide concentration, indicating that this is a dose-dependent relationship with delta-peptide and the intestinal tissue within the ligated ileal loop. The level of volume accumulation in the closed ileal loops for dose-dependent treatments may be observed in Figure 2-4D.

**Action of discrete delta-peptide species and negatives**

Interestingly, although E23ox is one of the more serum-stable variants of EBOV delta-peptide, there are many other peptide species of varying amino acid length as mentioned previously, with the C-terminal region being conserved (Figure 2-5A). To that end, we tested four total variants of EBOV delta-peptide: E15ox, E17ox, E23ox, and E40ox. Previously, we determined that the peak peptide activity occurs 9-12 hours after introduction of the peptide to the closed ileal loop. To test the discrete delta-peptide species, we chose a singular time point of 12 hours to assess the ability of the peptide to induce diarrheal syndrome. At a concentration of 100 µM, we found that E40ox, E23ox, and E17ox were all able to exert similar activity in the ileal loops they were injected into, resulting in diarrheal syndrome (Figure 2-5B). E15ox, however, was not able to induce gastrointestinal distress and the Loop Ratio was similar to the negative control (Figure 2-5B).

In previous studies, the reduced version of E23 (E23red) was shown to have very little pore-forming ability *in vitro*. In order to further test the necessity of peptide secondary structure for E23ox to retain activity, two strategies were employed to disrupt the secondary structure. The first approach was to simply scramble the sequence of E23 before oxidizing the cysteines. The second approach was to covalently alkylate the cysteines so that a disulfide bridge cannot be formed; this was
accomplished by reacting the cysteines with iodoacetamide and purifying the reaction product by HPLC and confirming the product by MALDI-MS. The scrambled sequence, E23(scr)ox, had less activity compared to E23ox but was still able to induce diarrheal syndrome (Figure 2-5C). The alkylated version, E23alk, also showed a reduction in Loop Ratio, but there was still an elevation in fluid accumulation (Figure 2-5C). These two experiments suggest that amino acid composition is likely as important as peptide secondary structure. This is a common feature of membrane-active peptides. In order to further validate the diarrheal pathology model being used, we also tested an unrelated, inert peptide called ONEG to demonstrate that introduction of the peptide alone is not enough to induce gastrointestinal distress (Figure 2-5C). Amino acid sequences for the peptides used in this section are shown in Figure 2-1A and Figure 2-5D. The level of volume accumulation in the closed ileal loops for the above described variants of the delta-peptides and negatives treatments may be observed in Figure 2-5E.

**Observing delta-peptide effects through histological analysis**

Although Loop Ratio is a powerful metric for determining diarrheal syndrome via fluid accumulation, secondary metrics are necessary to further confirm the level of gastrointestinal distress and elucidate more details about the mechanism of the peptide. To accomplish this, the majority of the resected ileal loops from the previous sections were fixed, paraffinized, and stained for relevant cellular markers. The first stain used was a Periodic Acid-Schiff stain, which is able to identify mucin-producing Goblet cells within the villi of the small intestine. After introduction of the peptide to the closed ileal loop, fluid accumulation occurs and begins to expand and stretch the intestinal tissue to its capacity. This results in the shortening of villi and crypts, as well as a massive depletion of mucin from Goblet cells into the lumen of the small intestine, likely to protect the small intestine from the damaging inflammatory conditions as indicated by the PAS stains. This depletion is seen as early as the 6-hour time point, and peaks during the 9- and 12-hour time points along with the Loop Ratio; pictured here is a snapshot of the villous structure and a visualization
of how the PAS stain appears using loop tissue from the 12-hour time point (Figure 2-6A). The Goblet cells are stained in bright magenta. In order to quantify this effect, the number of Goblet cells per villi were counted from representative samples from each experimental group of n=8 and then compared (Figures 2-6B). Additionally, architecture damage scoring was done to assess the extent of the damage done to the small intestine. This was accomplished by using a standardized scale to assess damage which served as a rubric for damage scoring, and a visual guide can also be seen in this panel (Figure 2-6C). The data for individual time points were then plotted to make histograms for all of the aforementioned metrics at the different concentrations of delta-peptide used. (Figure 2-6D). Representative cross-sections at 10X and 20X magnifications are shown in Figure 2-6E, on the left and right panels respectively. Histological analysis was also conducted on the dose-dependent studies mentioned previously. The number of Goblet cells per villi were counted for each experimental group and the two histopathological scores can be seen in Figure 2-6D for several time points and concentrations. The same scoring rubric was used to assign histopathological scores to each stained intestinal cross-section.

Fluorescent staining was also done on the loops using WGA-AF488 to stain for Goblet and Paneth cells and DAPI as a nuclear counterstain. Similarly, in Figure 2-6F, it is possible to visualize the fluorescent staining at 10X and 20X magnifications, on the left and right panels respectively. Using this technique, it is also possible to observe the differences in volume comparing negative control cross-sections of the mouse small intestine to scale by using ImageJ to make composite, stitched loop images (Figure 2-6G). A stain with the TAMRA-phalloidin as mentioned in the Methods section for Chapter 2 can be seen in Figure 2-6H. Here, even more detail is revealed to show the full architectural changes elicited by the EBOV delta-peptide, E23ox.

**Analyzing the genome-level responses to E23ox**
The advent of RNASeq has been a powerful advancement in genomic and transcriptomic analysis. In order to understand the effects of the delta-peptide on the cells of the small intestine, we extracted the genomic RNA from intestinal samples that were treated with 100 µM E23ox and with the negative control (0.025% AcOH) at the 6- and 9-hour time point using a Qiagen RNA Extraction kit. The transcriptomes of the ileal loops were converted to cDNA and aligned to the reference genome available for BALB/C mice. A volcano plot for the 6-hour time point shows statistically significant genes (by q-value) versus the natural log of the fold change in abundance (beta value) and can be seen in Figure 2-7A where each point represents an individual gene and several of the most significant genes have been labeled. The most upregulated genes are on the right, the most downregulated genes are on the left, and most statistically significant genes are near the top. Additionally, further analysis was done to confirm that statistical significance derived from q-values within this analysis also correlated with statistical significance indicated by p-values as well (Figure 2-7B). Gene Set Enrichment Analysis (GSEA) was done using previously established gene clusters provided by the Kyoto Encyclopedia of Genes and Genomes (KEGG), Biocarta, GO, and Reactome. Several gene set clusters were differentially affected on a statistically significant level if E23ox was administered. Differential gene set expression between the E23ox group and the negative control group may be visualized in Figure 2-7D, where the gene heatmap (Figure 2-7C) shows the genes which are most upregulated and downregulated when comparing an E23ox-treated mouse against a control at the 9-hour time point. The gene set families include apoptosis, adhesion molecules, cell cycle, cytokine signaling axis, antiviral mechanisms, TLR signaling, and the proteasome among many others. These enrichment plots can be visualized in Figure 2-7D, the majority of which show gene upregulation correlated with E23ox treatment. Each vertical line on the cluster analysis represents a singular gene associated with that specific gene set cluster, with both the 6- and 9-hour time points plotted in each GSEA family.
Many of these differential upregulations of genes are in accordance with previous observations within this study and the following genes are among the most differentially expressed when E23ox treatment occurred. Apoptotic genes such as Caspase-9 and Caspase-7 may be upregulated in response to cellular damage induced by E23ox\textsuperscript{83,84}. Adhesion molecules such as Claudins are highly upregulated as the tissue architecture is stretched to its limits due to fluid accumulation\textsuperscript{85}. As these cellular stresses are being exerted, the gene WEE1 is upregulated heavily to stop cell cycle progression\textsuperscript{86}. Immune signaling also occurs through TLR3 upregulation, which triggers a pro-inflammatory response\textsuperscript{87}. CCL25 is upregulated and leads to the development of T-cells and CCL20 is upregulated and induces chemotaxis of dendritic cells, both of which are indicative of immune cell infiltration\textsuperscript{88,89}. IL-15 is a cytokine that is upregulated in E23ox-treated mice and leads to the activation and proliferation of Natural Killer cells, normally dispatched against virally-infected cells\textsuperscript{90}. Additionally, several members of the Tumor Necrosis Factor (TNF) family and Interferon receptors are also upregulated, again confirming a type 1 immunologic response which leads to inflammatory conditions\textsuperscript{91,92}. Proteasome complex activators of the PSME and PSMB families are also upregulated in order to potentially confine inflammatory signaling\textsuperscript{93,94}. These activators are also likely the result of upregulated apoptotic genes\textsuperscript{93,94}. Additionally, there are also cytokines and receptors which are not canonically inflammatory being upregulated. The IL-22 receptor is upregulated and this signaling axis has been shown to aid in mucosal tissue architecture repair\textsuperscript{95}. IL-10 levels are also increased, and this may aid in controlling the production of pro-inflammatory cytokines as well as promoting the survival of B-cells\textsuperscript{96}.

**Discussion**

Our findings suggest that the EBOV delta-peptides can induce severe gastrointestinal pathology. These results have many implications beyond this study as well. The delta-peptides may contribute to the burden of diarrheal syndrome as well as other effects during active EBOV infections. Further exploration will require additional animal models that assess peptide activity on different organ
systems. Aiding in the pathogenesis of EBOV infections may also classify the delta-peptides as novel virulence factors. Although EBOV has a specific host range for infection and viral propagation, delta-peptides are not species-specific. They are membrane-interacting peptides which can insert into many different types of lipid bilayers. They function in a dose-dependent manner in vivo and many discrete delta-peptide species are able to elicit the same response. Severe fluid accumulation and extensive damage to the villous architecture is observed hours after administration to the closed ileal loop. Genome-level responses to E23ox show that there is a clear pro-inflammatory response that is elicited as well as tissue damage-associated responses. Furthermore, the in vitro studies using the ionophores Indo-1, AM and Sodium Green in Chapter 1 exemplify the ability of the peptide to form ion channels in the membranes of CHO cells, which may be the cause of the diarrheal pathology. EBOV delta-peptides act as enterotoxic viroporins and may contribute to the burden of morbidity and mortality of EBOV infections.
**Figure 2-1: All delta-peptide sequences used in this study**

This sequence table aligns all sequences of the delta-peptide used in this study. The top six peptides represent the native EBOV delta-peptides in various amino acid lengths starting the E40 and ending with E18n. The two cysteines are displayed in blue to designate where the disulfide bridge occurs. Below the top section are eight peptides which comprise the mutants, scrambled, and click-chemistry modified variants of E23. Amino acids that deviate from the native sequences are shown in red. The following five peptides represent the mutants of E40 used in this study. Finally, the bottom five sequences represent the delta-peptides formed by related filoviruses Reston virus (R25 and R42) and Cuevavirus (LLOVIU).
Figure 2-2

A

1. Midline incision
2. Expose intestine
3. Ligate loop
4. Inject loop
5. Suture mouse

B

<table>
<thead>
<tr>
<th>Surgery complete</th>
<th>Necropsy and ileal loop resection</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Hours</td>
<td>9 Hours</td>
</tr>
<tr>
<td>12 Hours</td>
<td>24 Hours</td>
</tr>
</tbody>
</table>

**Loop Ratio:**

\[
\text{Weight of the loop (g)} + \text{Length of the loop (cm)}
\]

<table>
<thead>
<tr>
<th></th>
<th>Normal Loop Ratio:</th>
<th>Elevated Loop Ratio:</th>
<th>Diarrheal Loop Ratio:</th>
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<tbody>
<tr>
<td></td>
<td>0.03 - 0.04</td>
<td>0.05 - 0.06</td>
<td>0.07+</td>
</tr>
</tbody>
</table>
Figure 2-2: Optimization and validation of the murine diarrheal pathology model used

(A) The roadmap for each surgical procedure using BALB/C mice. The surgery starts with a midline incision, whereby the small intestine can be exposed. Surgical silk is then used to ligate two sections of the small intestine just below the caecum in order to make a closed ileal loop. The lumen of the ligated loop is then injected with 100 µL of volume to fill up the loop and the syringe is removed carefully. The intestines are then placed back into the peritoneal cavity and the mouse is then sutured until the necropsy takes place. (B) The timeline for necropsy post-surgery is seen here. After the surgery is complete, the mouse is necropsied at pre-determined time points of 6, 9, 12, and 24 hours after introduction of the delta-peptide or other experimental groups to the intestinal lumen of the ileal loop. The ileal loop is then resected and weighed (g) and measured (cm) to determine the level of fluid accumulation via the Loop Ratio metric. We found that normal Loop Ratios without diarrheal pathology are around 0.03 to 0.04, anything that is more than double this number is what we consider diarrheal syndrome. Diarrheal syndrome will be denoted by a dashed line on each plot hereinafter. (C) Cholera toxin (CTX) at a concentration of 1 µg/loop was able to elicit diarrheal pathology at almost all time points tested. Statistical significance was observed at

![CTX vs. Control](chart.png)

- **0.025% AcOH**
- **CTX (1 µg/loop)**
the 6-, 9- and 12-hour time-points by t-tests performed using Graphpad Prism. For this plot and all subsequent plots, significance is designated as follows: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Figure 2-3

A

6 Hours

9 Hours

12 Hours

24 Hours
Figure 2-3: Effects of the EBOV delta-peptide on the small intestine within a closed ileal loop
(A) When testing the delta-peptide in the ileal loop compared to vehicle of the peptide as the negative control (0.025% AcOH) and 1 x 10^7 CFU *V. cholerae* at a sublethal dose as indicated by the original paper as the positive control, it is apparent that the EBOV delta-peptide E23ox is able to cause severe diarrheal syndrome. These results are significant at all time points observed; t-tests were conducted using Graphpad Prism. (B) The peak activity of the peptide can be visualized when plotting the Loop Ratios as a function of time point. Here, we observe that the diarrheal syndrome peaks at the 9- to 12-hour timepoints after introduction of the delta-peptide to the ileal loop. It is also able to outperform *V. cholerae* within the initial 24-hour observation window chosen. However, by 24 hours, the diarrheal pathology induced by the delta-peptide begins to subside. The mice that had *V. cholerae* administered to the ileal loops are likely to experience further diarrheal pathology if the bacteria were allowed to replicate for longer. (C) The fluid accumulation indicative of diarrheal pathology can been seen within the resected ligated ileal loops in this panel. These images have been chosen as they are representative of the entire experimental group they have been chosen for. Throughout the time course, the 0.025% AcOH negative controls look normal and there
is no mechanistic stress on the intestinal tissue. The *V. cholerae* group ileal loops appear to increase in fluid accumulated within the loop, especially at the 12 hour time point. The delta-peptide shows signs of fluid accumulation starting at the 9 hour time point and the tissue also appears to be under mechanical stress since it is stretched to its capacity limits. For these plots and all subsequent plots, significance is designated as follows: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Figure 2-4

A

6 Hours

9 Hours

12 Hours

24 Hours

E23ox Concentration (µM)

E23ox Concentration (µM)
B

E23ox (9 & 12 Hours)

Loop Ratio

0.00
0.04
0.08
0.12
0.16

0.025% AcOH
10 µM E23ox
20 µM E23ox
50 µM E23ox
100 µM E23ox
200 µM E23ox

✱✱✱✱✱
✱✱✱✱
✱✱
ns

E23ox Dose Dependency

C

E23ox Dose Dependency

Loop Ratio

0.00
0.04
0.08
0.12
0.14

6 Hours
9 Hours
12 Hours
24 Hours

0.025% AcOH
10 µM E23ox
20 µM E23ox
50 µM E23ox
100 µM E23ox
Figure 2-4: Analysis of concentration dependence of the delta-peptide in murine small intestines

(A) Testing the delta-peptide in the ileal loop at different concentrations compared to eluent of the peptide as the negative control (0.025% AcOH), it is apparent that the EBOV delta-peptide E23ox is able to cause severe diarrheal syndrome at several concentrations. Several delta-peptide treatment concentrations were statistically significant at different time points when compared to the negative control; t-tests were conducted using Graphpad Prism. (B) When the peak time points (9- and 12-hour time points) are aggregated, the results show that delta-peptide induces diarrheal syndrome in a stepwise, concentration-dependent manner. Here, a multiple comparisons ANOVA test was used to show statistical significance at the 20, 50, 100, and 200 µM concentrations compared to the control. (C) In this panel, as done in the previous figure, the Loop Ratios derived from the different time points are plotted as a function of the time point. Here, we observe that the diarrheal syndrome is elicited by the delta-peptide E23ox after introduction of the delta-peptide to the ileal loop at several concentrations. However, as seen previously, by 24 hours the diarrheal pathology induced by the delta-peptide begins to subside. Here, a modified chi-square test was used to show
significance at 20, 50, and 100 µM concentrations of E23ox. (D) The fluid accumulation indicative of diarrheal pathology can be seen within the resected ligated ileal loops in this panel. These images have been chosen as they are representative of the delta-peptide action for that particular time point. Fluid accumulation is apparent at several time points and concentrations, indicating that the delta-peptide is a potent enterotoxin even in small quantities. For these plots and all subsequent plots, significance is designated as follows: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Figure 2-5

E40ox

E23ox

E17ox

E15ox
Discrete Delta Peptides (100 μM - 12 Hours)
Figure 2-5: Testing discrete delta-species and peptide negatives within the murine diarrheal pathology model
(A) Shown in this panel are the different native sequences of the delta-peptide truncated from the starting 40 amino acid variant used in the murine diarrheal pathology model. All peptides here also show the disulfide bridge between the two cysteines, indicating the oxidized version of the peptide.

(B) The EBOV delta-peptide variants were tested at the 12-hour time point at a concentration of 100 µM, previously established as one of the time points of peak enterotoxic activity elicited by E23ox. Statistically significant fluid accumulation (determined by t-testing) is indicated by Loop Ratio for E40ox, E23ox, and E17ox. However, E15ox appears to have lost activity likely due to the loss of a large hydrophobic motif on the N-terminal side of E23ox. (C) This figure shows two E23ox variants specifically designed to disrupt structure in order to disrupt function. However, the scrambled variant still showed activity and E23alk which had both cysteines alkylated such that disulfide bridge formation was not possible also showed moderate activity. An unrelated inert peptide (ONEG) was also tested at this stage to confirm that random peptide presence alone was not enough to induce diarrheal pathology. For these plots and all subsequent plots, significance is designated as follows: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. (D) The sequences for all delta-peptide variants used in the murine diarrheal pathology model are shown and aligned. (E) The fluid accumulation indicative of diarrheal pathology caused by the peptides mentioned previously can be seen within the resected ligated ileal loops in this panel. It is apparent that three variants (E40ox, E23ox, and E17ox) are all able to induce severe diarrheal pathology.
Figure 2-6

A

0.025% AcOH

100 μM E23ox

12 Hours
Small Intestine Damage Inflammation Scores

**Crypt and villous architecture**
0 = Normal
1 = Irregular
2 = Shortening of villi
3 = Some villi completely lost, crypt length increased

**Goblet Cell Loss**
0 = normal/<10% loss
1 = 10-25%
2 = 25-50%
3 = >50%

Histology Scoring Examples

Architecture Scoring

Goblet Cell Loss Scoring
Figure 2-6: Histopathological analysis of murine small intestine tissue in response to E23ox

(A) The PAS stain makes the target of the stain (Goblet cells) a bright magenta color as seen in this panel using murine smallintestinal tissue from the 12-hour time point. The tissue on the left is from a mouse which was given the negative control (0.025% AcOH) and the villous architecture looks to be intact. The tissue on the right is from a mouse which was given E23ox at a concentration of 100 µM and the mechanical stress from fluid accumulation and shortening of the villi are both apparent. This is accompanied by depletion of Goblet cells. (B) The quantification of the data shows that Goblet cells are depleted in mice given E23ox. The left column represents the data generated from the initial ileal loops in Figure 2-3; the right column represents the data generated from the ileal loops done to determine concentration dependence in Figure 2-4. There are several time points of concentrations of E23ox that result in a statistically significant depletion (by t-testing) compared
to the negative control (0.025% AcOH). For these plots and all subsequent plots, significance is designated as follows: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. (C) A scoring rubric and visual guide to score the architecture loss and Goblet cell loss. In the visual guide, loss of architecture is largely designated by the shortening of villi, and for Goblet cell loss, yellow arrows are placed on positively stained Goblet cells (found along the villi, the positively stained cells in the crypts are Paneth cells)\textsuperscript{80,97}. (D) The scoring was then plotted as a function of time point and concentration and the score quantification for architecture loss and Goblet cell depletion can be seen in this panel. The statistically significant differences in scoring also are in accord with the Loop Ratio scores generated in the earlier portion of this chapter. (E) Representative cross sections at 10X (left) and 20X (right) can be seen in this panel highlighting the distribution of positively PAS-stained Goblet cells between the negative control (0.025% AcOH), the delta-peptide at 100 µM, and \textit{V. cholerae} at 1 x 10\textsuperscript{7} CFU. (F) Using immunofluorescent staining, WGA-AF488 is able to stain for Goblet and Paneth cells and DAPI is used as a nuclear counterstain. Representative cross sections at 10X (left) and 20X (right) can be seen in this panel. This stain was not used for scoring. (G) Immunofluorescent images are also conducive to photo-stitching by ImageJ to make composite, stitched loop images such that the images of the loops can be compared to scale with each other. Here, with loops used from the mice used in Figure 2-3, we see at both the 9- and 12-hour timepoints that the fluid accumulation induced by delta-peptide is apparent and severe. (H) Adding TAMRA-phalloidin to the previous staining mixture reveals more architectural details on the effects of E23ox on the small intestine.
Figure 2-7

A
Figure 2-7: Analyzing the genomic- and transcriptomic-level responses to E23ox in ileal loop tissue
(A) In this panel, a volcano plot was generated using the transcriptomes of the ileal loop tissue for both the negative control and E23ox-treated mice. A volcano plot for the 6-hour time point shows statistically significant genes (by q-value) versus the natural log of the fold change in abundance (beta value), where each point represents an individual gene and several of the most significant genes have been labeled. The most upregulated genes are on the right, the most downregulated genes are on the left, and most statistically significant genes are near the top. (B) This figure is an internal analysis of one particular GSEA cluster of genes from KEGG, it is done to confirm that the statistical significance values derived from the q-values within the analysis also correlate to statistical significances indicated by p-values for the same. (C) Differential gene expression between the E23ox group and the negative control group can be individually visualized in this panel as the most upregulated (right) and most downregulated genes (left) by averaging. (D) Here, stacked 6- and 9-hour time point GSEA plots can be seen using several gene clusters and several sources of these gene set families. The majority of these enrichment plots show gene upregulation correlated with E23ox treatment. Each vertical line on the plot represents a singular gene associated with that specific gene set cluster, with both the 6- and 9-hour time points plotted in each gene set plot.

CHAPTER 3: Engineering and synthesis of a E23ox antigen to immunize rabbits and produce a custom high-affinity binding antibody against EBOV delta-peptide

Introduction

In Chapter 2, we established that several EBOV delta-peptides induce gastrointestinal distress similar to enterotoxin-producing *V. cholerae* or purified cholera toxin (CTX) in a murine diarrheal pathology model. We may be able to consider them to be a small family of previously uncategorized virulence factors. Virulence factors have long been the target of therapeutic agents in filovirus
infections, with VP35 and glycoprotein (GP) being the primary targets\textsuperscript{98-101}. Antibodies against these targets have resulted in protective effects, both in prophylactic and therapeutic settings, suggesting that viral subunit targets may be crucial for preventing and treating EVD pathogenesis\textsuperscript{102,103}. As such, we approached the EBOV delta-peptide as a novel druggable target which is a conserved portion of the EBOV genome. A broadly neutralizing antibody may be able to bind and reduce the activity of the delta-peptide. This idea has not been tested, which represents a gap in the field. An anti-EBOV delta-peptide antibody may have future implications in diagnostic, prophylactic, and therapeutic settings.

Short peptides are usually not good immunogens unless they are conjugated to a carrier protein\textsuperscript{104}. Carrier proteins have long been used to increase the immune visibility and processing of peptide antigens\textsuperscript{105,106}. Carrier proteins such as Keyhole Limpet Hemocyanin (KLH), bovine serum albumin (BSA), or ovalbumin (OVA) are often used to conjugate smaller haptens (1-5 kDa) for specific antibody production\textsuperscript{107-110}. The EBOV delta-peptide E23ox is a roughly 2 kDa peptide, which fits this hapten description. However, special consideration must be given to the antigen-carrier complex to select for the best epitopes to optimize binding ability to the native unconjugated antigen. In this work, the antigen is the E23ox variant of the EBOV delta-peptide. E23ox must be developed into an antigen which can be processed and presented by dendritic cells to B cells, which can then undergo a somatic hypermutation process for the development of secreted high-affinity neutralizing antibodies (IgG) against native delta-peptide\textsuperscript{111}. The sequence of E23ox contains multiple lysine and cysteine residues that are critical for activity. Yet, these particular amino acids interfere with traditional peptide-carrier coupling strategies. Thus, a modified peptide will be used here to introduce a new chemically reactive group and a non-traditional coupling technique will be used to convert the EBOV delta-peptide into a non-structural subunit antigen.

Several of the current vaccine candidates being developed for EVD are EBOV glycoproteins or other non-structural proteins in an adenovirus VSV-platform used to generate antibodies\textsuperscript{112-115}. 
There are also a small number of non-structural protein vaccine candidates as well. Some of these also use the adenovirus platform as a killed virus strain\textsuperscript{116}. Here, since our antigen is a peptide, we will not use an adenovirus platform to generate an antigen for antibody production. Our non-replicating subunit antigen will simply be a modified variant of E23ox conjugated to KLH using a heterobifunctional crosslinker. There is precedence for this type of conjugation strategy, however it is uncommon\textsuperscript{117–122}. Our approach may be a novel strategy in alternative peptide-carrier protein antigen engineering.

Once the modified E23ox peptide is successfully conjugated to KLH using the heterobifunctional crosslinker, we can use the antigen to immunize rabbits to develop high-affinity antibodies to the EBOV delta-peptides. Using a set immunization schedule and adjuvants, the rabbits which are immunized will develop antibodies to the antigen and the rabbits can be bled at predetermined intervals to collect the antisera. The antisera can then be tested for the presence of specific antibodies against E23ox and the IgG can subsequently be purified for preclinical testing and evaluation. The antibodies we obtain in this work may give us insight into whether the EBOV delta-peptides are druggable targets in EBOV prophylactic and therapeutic development.

**Materials and Methods**

*Preparation of a modified peptide antigen*

Although there are many discrete delta species of varying amino acid length, the oxidized 23 residue version (E23ox) was chosen as the antigen of interest. Normally, numerous copies of the peptide of interest are conjugated via lysine linkage to a carrier protein such as Keyhole Limpet Hemocyanin (KLH). However, this peptide has four naturally occurring lysine residues as well as an internal cystine disulfide linkage which may provide secondary structure crucial for activity. Accordingly, non-traditional coupling strategies must be employed in order to maintain the
structural integrity of the antigen of interest. KLH remained the carrier protein of choice, however, since it is an industry standard and is a versatile, immunogenic protein.

The first quandary that had to be overcome was the reactivity of the peptide to the carrier protein without disrupting the structure of the peptide itself; click chemistry may be able to resolve this issue. The basic principle of click chemistry is that an azide-containing group can be covalently reacted to a cyclooctyne group in a very fast reaction that uses the ring strain of the cyclooctyne to create a conjugate of the two reactants (Figure 3-1A). To that end, a modified version of the E23 peptide was synthesized by Biosynthesis, Inc. with a double glycine linker and an azide-containing lysine (Figure 3-1B) at the N-terminal of the peptide, making it a 26-residue peptide which we termed azidoE23ox (sequence in Figure 2-1). The peptide was then oxidized as mentioned previously in the Methods section of Chapter 2. Once the oxidation of the cysteines was complete and the excess DMSO had been removed from the peptide, this peptide was first tested in the murine diarrheal pathology model to ensure antigenic fidelity, and then the peptide reacted with DBCO-maleimide eluted in DMF (Figure 3-1C). This reaction allowed the peptide to have a new reactive group in the covalently-linked maleimide using click chemistry.

**Preparation of the carrier protein using a heterobifunctional crosslinker (SPDP)**

Next, the carrier protein (KLH) needed to be prepared for peptide coupling (Figure 3-2). In order to achieve this, lysine residues on the KLH protein were reacted with a heterobifunctional crosslinker called succinimidyl 3-(2-pyridyldithio) propionate (SPDP) where the crosslinker was added at a 500:1 molar ratio to KLH. The buffer used for all reactions in this portion was PBS-EDTA pH 7.5 (100 mM NaPO₄, 150 mM NaCl, 1 mM EDTA, 0.02% NaN₃). There are roughly 300-600 lysines available on each KLH protein for coupling. Previously, a heterobifunctional crosslinker termed SMCC had been used to conjugate haptens to KLH, however, our strategy employs a different crosslinker and chemical linkages. SPDP has two reactive groups: an N-
Hydroxysuccinimide (NHS) ester and a pyridyldithiol which react to amino groups and sulfhydryl groups respectively. The NHS ester and the amino group from the surface lysine residues on KLH react to form a stable amide\textsuperscript{126}. This allows KLH to have a new accessible reactive group (pyridyldithiol) to covalently couple the antigen of interest. Once the protected sulfhydryl group on the unreacted side of SPDP is de-protected by TCEP (a reducing agent), it releases a byproduct which allows for reaction tracking, and the maleimide-containing peptide is ready to be coupled to KLH. The maleimide on the peptide will react with the sulfhydryl group from the SPDP-linked KLH to form a stable thioether bond (also accomplished using the PBS-EDTA pH 7.5 buffer). It should be noted that during these intermediary reaction steps, buffer exchanges are done 3-5X using Zeba Desalting columns to get rid of excess reactants and byproducts.

**Conjugation of the maleimide-containing azidoE23ox to the SPDP-linked KLH**

The maleimide from the modified peptides and the open sulfhydryl groups on the SPDP-linked KLH will then react to covalently couple the peptide to the carrier protein. There are several surface-available lysine residues on KLH which increases the likelihood of conjugating several peptides to the carrier protein, thereby increasing the probability of an immune response during antigen processing\textsuperscript{124,125}. Several test reactions were conducted at a small scale using a TAMRA-maleimide and measuring the increase in absorbance at 541 nm. The reaction was confirmed to have taken place by HPLC and MALDI mass spectrometry. Interestingly, during this reaction, the KLH crashed out of solution and formed crosslinked aggregates, but the solubility of KLH is known to be low\textsuperscript{127}. Fortunately, KLH which has crosslinked and aggregated is still able to be used as an immunogen\textsuperscript{128}. In total, 5 mg of total antigen was produced for immunization in rabbits to produce custom antibodies. Once the final conjugate was purified, buffer-exchanged, and aliquoted, the antigen-coupled KLH was shipped off to ProSci (Poway, CA) for immunization in two rabbits, in the pursuit of high-affinity neutralizing antibodies against E23ox. In this section, the same pH 7.5 PBS-EDTA buffer used previously was made without the use of sodium azide, as that is toxic to
animals. This stage of the project was done in coordination with our collaborators at Autoimmune Technologies (AIT) (New Orleans, LA), as they are experienced in antibody generation. A reaction map outlining all the steps in a visual fashion can be seen in Figure 3-3.

**Using click chemistry to make a dye-labeled variant of E23ox**

The remaining azidoE23ox peptide which was not used in the previous KLH conjugation strategy was used to engineer a dye-labeled variant of E23ox. A dye-labeled variant may have utility in understanding binding to vesicles and cells *in vitro*, tracking where the peptide goes in the ileal loop using the murine diarrheal pathology model *in vivo*, and potentially using the peptide in immunoblotting or ELISAs. The peptide was reacted with Click-iT™ Alexa Fluor™ 555 sDIBO Alkyne and Click-iT™ Alexa Fluor™ 555 DIBO Alkyne over the course of 20 days and the reaction was tracked using HPLC. Both the 280 nm and 555 nm wavelengths were used to track the reaction as the 280 nm channel can be used to detect peptide and the 555 nm channel would indicate dye presence on the peptide. Once the reaction was complete, the fraction collection function of the HPLC was used to collect the appropriate peaks representative of the AF555-labeled E23ox and these samples were dried down to remove the HPLC buffers. Something to note about these experiments is that one of the dyes was discontinued as the large-scale reaction was going forward. As such, the cyclooctynes of the dyes are slightly different from each other (DIBO vs. sDIBO) and resulted in an extra peak in the HPLC traces as they have slightly different retention times (Figure 3-4). The dried down peptide was then reconstituted in 0.025% AcOH and then used as needed. An example of this peptide being used *in vitro* can be seen in Figure 1-5C and 1-5D.

**Immunization of rabbits using our antigen to develop custom antibodies**

Two rabbits were immunized using our antigen using an IACUC-approved protocol overseen by a team at the Prosci lab. KLH-based conjugates have been used successfully in rabbits previously to
develop high affinity antibodies. In the immunization schedule as communicated by Prosci, both Complete Freund’s Adjuvant (CFA) and Incomplete Freund’s Adjuvant (IFA) are used to boost the immune response, which has also been long established and tested (Figure 3-5)\textsuperscript{129,130}. Pre-immunization antisera was collected from the rabbits prior to the start of the immunization schedule to compare against the post-immunization antisera. After the initial immunization with CFA, all subsequent boosts use IFA and bleeds are taken in intervals to test for the presence of anti-delta-peptide antibodies. Production bleeds are spaced by two-week intervals and an immunizing boost is given to the rabbits one week before the two bleeds are done. Afterwards, the cycle is repeated with another immunizing boost; for this study, antisera from 19 production bleeds were collected over the course of a full year.

\textit{Testing reactivity of the test bleed antisera against the delta-peptides and antigenic components}

The test bleed antisera sample was concentrated from a small volume of blood taken from each of the two rabbits after three immunizations using the antigen that was engineered. This was shipped to us in New Orleans, LA from Poway, CA and delivered to our lab at Tulane University by AIT. In addition to the ELISA which Prosci conducted using unconjugated peptide which was also shipped with the antigen, we ran several Western blots using several EBOV delta-peptide variants (E14ox, E15ox, E17ox, E18n, E23red, E23ox, E40ox), unrelated peptide controls (ONEG, MelP5), EBOV delta-peptide amino acid mutants which were synthesized by Biosynthesis Inc. (sequences can be seen in Figure 2-1), other filovirus delta-peptides (R25, R42, LLOVIU) to test for cross-reactivity, and individual antigenic components (azidoE23ox, azido-E23ox-maleimide, unloaded KLH, and the KLH-SPDP-E23ox conjugate). This allowed us to make a preliminary binding profile for both rabbits without having to purify the antibody for an ELISA as antisera samples were limited at this stage. All peptides and samples were tested at a concentration of 250 µM to ensure a large visible band; the test bleed antisera samples (one from each rabbit) were treated as the primary antibody and a secondary HRP-linked antibody was used for visualization via chemiluminescence.
At this stage, the pre-immunization antisera samples were also tested in a more curtailed fashion to assess non-specific binding by Western blotting. The production bleed antisera samples were not used in this step.

Results

Preparation of a modified peptide antigen

A modified version of E23ox synthesized to maintain the structural integrity of the peptide antigen during the conjugation was first tested in the previously described murine diarrheal pathology model (Figure 3-6). This modified peptide (azidoE23ox) was able to induce similar levels of diarrheal pathology based on our Loop Ratio metric, which confirmed that our modifications did not disrupt activity. The azidoE23ox peptide was then reacted with a compound called DBCO-maleimide, which causes a reaction through click chemistry. This reaction was tracked using HPLC (Figure 3-7A) and the weight of the final product was first collected by fraction collection and then confirmed by MALDI-MS (Figure 3-7B). The final product was purified using HPLC fraction collection, the peptide was dried down under a vacuum, and then finally reconstituted in 0.025% AcOH. At this stage, the peptide was ready for conjugation to the KLH carrier protein.

Preparation of the carrier protein using a heterobifunctional crosslinker (SPDP)

The carrier protein (KLH) was prepared by reacting the surface available lysine residues with a heterobifunctional crosslinker called SPDP. The end of the crosslinker which contains the NHS ester reacts with the amine group of the lysine to create a stable amide. This allows for the release of a byproduct which needs to be removed through buffer exchanges. The unreacted end of the crosslinker is then deprotected by TCEP so that the reaction between the sulfhydryl and the maleimide can take place, forming a stable thioether bond. In this step, the release of a fluorescent byproduct can be tracked at 343 nm and was used as a metric for the number of washes necessary
during the buffer exchange; this can be visualized in Figure 3-8. The next step was to use a TAMRA-maleimide molecule to confirm the reactive capacity of the open sulfhydryl groups on the KLH protein; this allows us to quantify the number of molecules potentially conjugated to the prepared KLH-SPDP molecule (Figure 3-9A). At this stage, the carrier protein KLH was ready for conjugation to the azidoE23ox-maleimide peptide.

**Conjugation of the maleimide-containing azidoE23ox to the SPDP-linked KLH**

In order to confirm that the conjugation between the maleimide-containing peptide would take place with the sulfhydryl groups of the SPDP-linked KLH, test reactions were done using TAMRA-maleimide in order to rudimentarily quantify the number of peptides we may expect to get on the carrier protein. This quantification can be seen in Figure 3-9A, although these numbers are lower than anticipated. More importantly, this result signified that the reaction worked, and we are able to use the sulfhydryl-maleimide reaction to conjugate cargo to the KLH protein. The principle behind the reaction is that the maleimide on the peptide will covalently react with the freshly deprotected sulfhydryl groups on the SPDP-linked KLH, forming a stable thioether bond. After the test reactions with TAMRA-maleimide, the azidoE23ox-maleimide was then reacted with the SPDP-linked KLH and the reaction was tracked and confirmed using HPLC and MALDI-MS in several different ways using process of elimination and with the consideration that HPLC is sensitive to even small concentrations of molecular compounds (Figure 3-9B-E).

**Using click chemistry to make a dye-labeled variant of E23ox**

The azidoE23ox peptide which was not used to make the KLH conjugate was used to make a dye-labeled variant of E23ox to track the peptide fluorescently in different capacities. It is possible to visualize the retention time shift as the reaction moves to completion via HPLC. Since there was a product discontinuation in the middle of the reactions, a slightly different dye was used for the large-scale dye-labeling (DIBO-AF555 and sDIBO-AF555). This is apparent in the peaks shown
in the HPLC trace (Figure 3-4). However, all the peaks which were present on the 555 nm channel were selected for fraction collection and further purification. This delta-peptide dye-labeled variant was used in the previous chapter but was synthesized during this portion of the study.

**Testing reactivity of the test bleed antisera against the delta-peptides and antigenic components**

Immunization of two rabbits using our antigen to develop custom antibodies then occurred under the oversight of Prosci, a custom antibody company. An IACUC-approved adjuvanted immunization schedule was approved and regular immunizations took place over the course of one year, interspersed by production bleeds conducted on the rabbits to collect antisera. A total of 19 production bleeds were taken from each rabbit before the study ended. A test bleed was also taken after the first 3 immunizations before the start of the production bleeds to confirm immunoreactivity against the delta-peptide and to continue the immunizations. The test bleeds were first tested by Prosci themselves in an ELISA conducted at their labs using the native E23ox delta-peptide as the plate-coating antigen, which was sent as part of a kit of supplies that were required from our laboratory prior to the start of the immunizations (Figure 3-10E). Once the test bleeds were sent to our laboratory at Tulane, we conducted much more extensive immunoreactivity tests on EBOV delta-peptide variants (E14ox, E15ox, E17ox, E18n, E23red, E23ox, E23scr, E40ox), unrelated peptide controls (ONEG, MelP5), EBOV delta-peptide amino acid mutants which were synthesized by Biosynthesis Inc. (sequences can be seen in Figure 2-1), other filovirus delta-peptides (R25, R42, LLOVIU) to test for cross-reactivity, and individual antigenic components (azidoE23ox, azido-E23ox-maleimide, unloaded KLH, and the KLH-SPDP-E23ox conjugate) by Western blotting (Figure 3-10 A-D). This allowed us to formulate a binary (non-quantitative) binding profile of the antibodies derived from each rabbit. Interestingly, both rabbits appear to process the antigen differently and this resulted in differential binding capacity as noted in the Western blot binding profiles for both rabbits (Figure 3-11). In short, the peptides are run on an SDS-Page gel and then
transferred to a nitrocellulose membrane using the Invitrogen iBlot transfer system. The membrane is then incubated overnight in the antisera diluted in PBS-dried milk (0.05% Tween) as the primary antibody incubation step. The following day, the primary antibody is washed away from the membrane using PBST (0.05% Tween) and an HRP-linked anti-rabbit immunoglobulin secondary antibody is added prior to development using a chemiluminescence kit and imaging the membrane on an appropriate imager. By Western blotting, the antibodies generated from both rabbits can bind several EBOV delta-peptide variants, several E23ox and E40ox delta-peptide mutants, and individual antigenic components. However, it does not appear to bind related filovirus delta-peptides generated by Reston virus and Lloviuvirus, indicating that there isn’t likely any cross-reactivity. It also did not bind peptide negatives such as ONEG and MelP5, which indicates high specificity for the EBOV delta-peptide sequences only. Additionally, the pre-immunization antisera did not have any reactivity to the delta-peptide.

Discussion

In this chapter, we describe a strategy in which the recently characterized EBOV delta-peptide, E23ox, was modified and conjugated to a carrier protein (KLH) using a non-traditional coupling technique using a heterobifunctional crosslinker (SPDP). Although this type of conjugation strategy has been briefly described in the literature, our approach uses a heterobifunctional crosslinker, SPDP, to conjugate a hapten to a carrier protein in a novel fashion\textsuperscript{117-122}. This conjugation strategy resulted in an antigen which was used to immunize rabbits with the optimism that they would be able to generate high-affinity antibodies against E23ox. The antisera which was received post-immunization contained antibodies that were reactive to several EBOV delta-peptide variants and the antigenic components when tested by Western blotting. Testing of the pre-immunization antisera suggests that there isn’t nonspecific binding, further validating our antigen as a viable immunizing antigen for EBOV delta-peptide.
This accomplishment of developing specific antibodies against the delta-peptides represents several novel endeavors in the field of EBOV science. This novel conjugation strategy was born out of necessity to preserve the native sequence of the peptide, which contains several essential lysine and cysteine residues. Generally, peptide antigens are specifically selected to have no lysines and only a single cysteine for ease of coupling to a carrier protein via peptide-cysteine to protein-lysine conjugation. For this work, we used an N-terminus-modified azide-containing E23ox variant. Click chemistry was used to react the azide-containing peptide with DBCO-maleimide in order to add a new, reactive functional group (maleimide) onto the peptide. The maleimide group was then used to conjugate the peptide to a carrier protein (KLH) which had been reacted with a heterobifunctional crosslinker (SPDP). The work in this chapter may lead to additional opportunities to develop novel therapeutics against EBOV, specifically targeting the delta-peptide, which may have a larger role in pathogenesis and virulence of EVD than previously thought. It may also have further considerations for EBOV vaccine development, though it will require much further exploration as the antigen is non-replicating. Finally, this strategy could represent a novel technique in custom antibody development against microbial haptens which are otherwise difficult to generate antibodies against.
Figures

Figure 3-1

A

B
Figure 3-1: The principles of click chemistry in antigen engineering

(A) This panel outlines the basic reaction between the leftmost compound, which contains the cyclooctyne necessary for click chemistry, and the azide (N$_3$) reactant in order to form two isomers (of equal chemical composition). (B) The modified lysine which contains an azide group which was added to the N-terminal of the E23ox peptide for click chemistry and further conjugation to KLH. (C) Dibenzylcyclooctyne (DBCO)-maleimide was then used to react the cyclooctyne portion of this compound to the azidoE23ox in order to add a reactive maleimide to the modified peptide.
Intricate topology of KLH
Molecular Structure

Figure 3-2: Topological structure of the carrier protein, keyhole limpet hemocyanin (KLH)

Keyhole limpet hemocyanin is a large multi-subunit metalloprotein used to carry oxygen found in
the hemolymph of a gastropod family of aquatic snails\textsuperscript{124}. It is very large in size (350-390 kDa) and
is commonly used to conjugate low molecular weight haptens to the protein to serve as an
immunogenic antigen\textsuperscript{107}. Furthermore, it is also phylogenetically distant to mammalian proteins\textsuperscript{107}.
This image is attributed to Gatsogiannis & Markl regarding keyhole limpet hemocyanin (KLH)\textsuperscript{131}. 
Figure 3-3

- **Step 1:**
  - React available lysines on mcKLH with a heterobifunctional crosslinker (SPDP)

  ![Figure 3-3 step 1](image)

- **Step 2:**
  - Reduce unreacted end of SPDP conjugated to KLH to expose open sulfhydryls

  ![Figure 3-3 step 2](image)

- **Step 3:**
  - React maleimide-containing E23ox to SPDP-linked KLH in a 1:1 ratio (w:w)

  ![Figure 3-3 step 3](image)

- **Step 4:**
  - Confirm reaction has taken place, purify the final product through buffer exchange washes, send off for antibody production

![Figure 3-3 step 4](image)

**Figure 3-3: A reaction map depicting each intermediate step in conjugating a modified E23ox to KLH**

In Step 1, the available lysine residues on the mariculture KLH (mcKLH) are reacted with a heterobifunctional crosslinker (SPDP). In Step 2, the unreacted end of SPDP is reduced using TCEP to expose open sulphhydryl groups on the crosslinker. In Step 3, the maleimide-containing E23ox will be reacted with the open sulphhydryl groups in order to conjugate the peptide to the carrier protein (KLH). The reaction will be tracked via HPLC and MALDI-MS as Step 4.
Figure 3-4: Using click chemistry to make a dye-labeled variant of E23ox

The wavelengths of 280 nm and 555 nm were used to track the reaction of azidoE23ox to DIBO-AF555 and sDIBO-AF555 as the 280 nm channel can be used to detect peptide and the 555 nm channel would indicate dye presence on the peptide. The left column indicates the HPLC traces for a mock reaction (unreacted) on Day 0 where the peptide is indicated on the 280 nm channel by a retention time of almost 24 minutes using this HPLC method. However, as expected, there is no signal from the 555 nm channel. By Day 20, the peptide had reacted with the dyes and were ready for fraction collection. On the 280 nm channel, there are three peaks. One represents unreacted peptide, one represents peptide reacted with DIBO-AF555, and the final peak represents peptide reacted with sDIBO-AF555. This is observable as only two peaks from the 555 nm channel overlap.
with the three peaks from the 280 nm channel. The two peaks from the 555 nm channel were selected for fraction collection by HPLC.
Figure 3-5: Outline of the immunization and bleed schedule for the rabbits immunized with the antigen

Prosci Inc. designed and oversaw the schedule for immunization of the rabbits using the antigen engineered earlier in this study. The schedule above shows the frequency of the antigen administered to the rabbits. Complete Freund’s Adjuvant (CFA) and Incomplete Freund’s Adjuvant (IFA) are used to boost the immune response as the adjuvants and bleeds are scheduled at least 7 days after a boosting immunization. In total, 19 such bleeds were collected.
Figure 3-6: Testing the modified peptide in the murine diarrheal pathology model

The azide-containing E23 peptide was oxidized to azidoE23ox and tested in the murine diarrheal pathology model in order to assess antigenic fidelity prior to carrier protein conjugation. Fortunately, the azidoE23ox maintained very similar loop ratios to native E23ox, indicating that this modified peptide retained the enterotoxic activity. As such, this peptide was used in antigen engineering in the steps outlined within this chapter. For these plots and all subsequent plots, significance is designated as follows: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Figure 3-7

A

- **Step 1:**
  - Oxidize azidoE23 – Confirmed by HPLC (characterized by 10-15 second shift in retention time)

- **Step 2:**
  - Click chemistry to conjugate DBCO-maleimide to the azide-containing lysine of azidoE23ox – Confirmed by HPLC and MALDI
Figure 3-7: Using click chemistry to add a maleimide group to a modified E23ox peptide

(A) The azidoE23 was first oxidized using the protocol described in the Methods section. The oxidation is characterized by a retention time shift from ~23.50 to ~23.85 minutes (roughly 20 seconds) in Step 1. The DBCO-maleimide is then reacted with the azidoE23ox and the HPLC shift is more drastic, which is depicted in Step 2. (B) When running the maleimide-containing E23ox on MALDI-MS, the expected molecular weight is 3540.2 g, and the trace shows a large singular peak at 3539.2 g. This indicates that the reaction worked and the maleimide group was added successfully to the peptide.
Figure 3-8: Monitoring the reaction between KLH and SPDP via byproduct tracking

KLH was reacted with a heterobifunctional crosslinker (SPDP) and prior to addition of the peptide, several buffer exchanges using Zeba desalting columns were done to ensure that there no inadvertent reactants in the reaction vessel. The unreacted end of the SPDP linked to KLH was deprotected using TCEP (indicated in the figure) and each subsequent buffer exchange of the conjugated product (CP) and the flow through (FT) were run on a Nanodrop 2000c to observe the 280 nm and 343 nm channels. SPDP releases a byproduct which can be read at 343 nm once the TCEP deprotects that end of the heterobifunctional crosslinker. Accordingly, once the TCEP is added to the reaction, the 343 nm absorbance spikes and then decreases after each buffer exchange for both CP and FT. Once the 343 nm signal approaches 0 Absorbance, the sample can be considered free of unwanted byproducts. The 280 nm channel shows that by the sixth wash that there is no more protein content left in the FT. Though there is protein content in the CP, there is a
significant and appreciable loss of total protein content from the buffer exchange steps. However, it is possible to remove the byproduct from the final conjugated product.
\[
\frac{A_{541}}{C} \times \frac{\text{MW of protein}}{\text{mg/mL protein}} = \frac{\text{Moles of dye}}{\text{Moles of protein}}
\]

<table>
<thead>
<tr>
<th>KLH-TAMRA TEST REACTIONS</th>
<th>(MOLES DYE/MOLES PROTEIN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIAL 1</td>
<td>negligible</td>
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<tr>
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<td>1.87</td>
</tr>
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<td>TRIAL 7</td>
<td>1.37</td>
</tr>
<tr>
<td>TRIAL 8</td>
<td>1.22</td>
</tr>
</tbody>
</table>
azidoE23ox-maleimide only

Reaction buffer supernatant
The reaction supernatant was not detected by MS in Free peptide (reaction) was.

Expected MW: 35402.2

Reference peptide: 35402.2 (ox)-monomeric
D

azidoE23ox-maleimide only

Final product aggregate
The final product was not detected by ESI in Free Peptide (reaction) was.

Expected MW: azidoE2(cy)-maleimide 35102
(A) A series of small-scale test reactions were done with the KLH-SPDP and a TAMRA-maleimide to see how many dye molecules can be conjugated to a single KLH protein. The absorbance of the dye at 541 nm is divided by the extinction coefficient of the dye (ε = 95000) and then multiplied by the molecular weight of the protein divided by the concentration of the protein. This results in a number indicating the moles of dye divided by the moles of protein. This calculation can represent the number of individual dye molecules on each KLH protein. (B) Once the large-scale reaction was started, the KLH crosslinked and aggregated, resulting in a slurry. To test if the reaction was completed, the reaction supernatant was run on HPLC to detect free peptide. A reference trace showing the retention time for ‘peptide only’ is also shown to indicate when the peptide should come off the column. There is no peak in the reaction supernatant, indicating that free peptide is not present and indirectly indicating that the reaction went forward. (C) The supernatants of the reaction mixture were then tested by MALDI-MS to determine presence of free peptide, here the reference peptide is an unrelated peptide (PHD108) to show where the peak should occur. This further confirms that the reaction went forward. (D) Once the KLH reaction mixture was allowed to settle and separate into the supernatant and gel phase, the gel phase of the reaction was then tested by HPLC to detect free peptide, again a reference peptide is run to illustrate what the retention time should be for that peptide. This trace indicates that there is no free peptide in the crosslinked aggregate, further confirming that the reaction went forward. (E) Finally, the gel phase of the reaction mixture was tested by MALDI-MS to detect free peptide in the crosslinked aggregate that had crashed out of solution. Here, a reference peptide again illustrates where the peak should be by molecular weight. However, the bottom two panels indicate no free peptide, further confirming that the reaction went forward and that there is no detectable free peptide in any portion of the reaction mixture. This suggests that the peptide is bound to the crosslinked aggregate KLH.
Figure 3-10

A
Figure 3-10: Testing reactivity of the test bleed antisera against delta-peptides and antigenic components

(A) Using the test bleed antisera as the source of the primary antibody in order to conduct Western blots proved to be useful as several delta-peptide variants were recognized (E15ox, E17ox, E23red, E23ox, and E40ox). The antibody was unable to recognize the delta-peptide variants E14ox, E18n, and E23scr. Furthermore, the antibody was unable to recognize two unrelated negative control peptides, ONEG and MelP5. As a note, ‘O’ indicates an older stock of E23ox and ‘N’ indicates a newly prepped stock of E23ox. These binding signatures were the same for both rabbits. (B) Using the same Western blotting technique, several delta-peptide mutants were tested and it appears that rabbit 22017 produces antibodies which are capable of binding more mutant variants of the delta-peptide than rabbit 22018. (C) With respect to delta-peptides which are produced by filoviruses related to EBOV, it appears that neither rabbit produced antibodies that are cross-reactive with R24ox and R42ox from Reston virus and LLOVIUox. The odd coloring of this Western blot is the
result of the imager using the autoexposure function to find a signal. (D) Individual antigenic components were then tested for reactivity with the antibodies from the rabbit antisera. Antisera from both rabbits appear to show reactivity to all antigenic components with varying intensity. The peptides azidoE23ox, azidoE23red, and azidoE23ox-maleimide are reactive and the carrier proteins unloaded KLH and E23ox-loaded KLH all appear to show banding. This indicates that several antibodies of the antisera repertoire are likely reactive to KLH as well as E23ox. (E) Finally, the ELISA from Prosci Inc. indicates a difference between the pre- and post-immunization antisera for both rabbits with respect to reactivity with E23ox. These results are all confirmatory for the previously described conjugation strategy and validates this engineered antigen.
Figure 3.11: Preliminary binding profiles of the antisera from two rabbits using Western blotting.
Using Western blotting, several peptides and proteins were tested for reactivity with the antibodies found in the test bleed antiserum collected from two rabbits. Although rudimentary, these binding profiles provide the sequences of all delta-peptide variants tested in this section and a color-code has been used to indicate binding ability. The amino acids depicted in different colors show the deviations from the native sequences of the EBOV delta-peptides. The darker green shows obvious binding by Western blotting to the peptide, which is highlighted, the lighter green indicates binding (with lighter bands), and the red indicates no apparent binding. Again, it appears that one rabbit (22017) is able to produce antibodies which are capable of binding more delta-peptide variants than the other rabbit (22018). However, this is not a quantitative profile and ELISAs will have to be conducted to more accurately define binding abilities.

CHAPTER 4: Purifying IgG and determining the in vitro therapeutic potential of the custom antibody against the delta-peptide in the context of synthetic vesicles and eukaryotic cells

Introduction

The development of a custom antibody against the EBOV delta-peptides opens several preclinical avenues for exploration. Antibodies against viruses and virus-specific antigens can be used as therapeutic options or as diagnostic tools\textsuperscript{132-141}. Furthermore, the antigen used to develop the custom antibodies may have further implications as a component of a vaccine. In order to test the applicability of the custom antibodies, they must first be purified from whole antiserum to an IgG fraction. The whole test bleed antiserum samples were used in the previous chapter as the source of the primary antibodies used in the Western blotting when testing the binding ability of the antibody against several delta-peptides and other components. However, there are several proteins which comprise whole antiserum and this in turn, may require a higher volume and concentration needed for the IgG to bind and recognize the EBOV delta-peptides.
The IgG fraction of antisera can be purified in several ways, one of the most common methods being affinity purification. A well-established method of affinity purification uses bacterial proteins (Protein A, Protein G, Protein L) to bind the Fc portion of the IgG molecules\textsuperscript{142,143}. This allows for a separation of IgG from whole antisera using Protein A/G/L coupled to agarose beads in a resin\textsuperscript{142,143}. Although this method is effective, it pulls down all the IgG in the antisera regardless of specificity to the targets of interest, the EBOV delta-peptides. This suggests that there will be IgG in the purified fraction that is not specific for the delta-peptides.

In order to reduce the concentration of non-specific IgG, a second round of higher stringency antigen affinity purification can also be conducted. We tested two ways of increasing specificity of the IgG. First, we used a modified version of the EBOV delta-peptide (azidoE23ox) coupled to DBCO-linked agarose beads to purify the delta-peptide specific IgG population out of the whole IgG population. Second, we used KLH coupled beads to remove the KLH-specific IgG, and leaving the remaining IgG with increased specificity for the delta-peptides. These methods can be used in combination with each other or as individual strategies.

Once the purified IgG fractions are obtained using a combination of the affinity purification processes, purity of the IgG fractions can be tested by running SDS gels, binding ability can be tested through ELISAs and Western blotting, and the therapeutic index can be determined by conducting certain \textit{in vitro} assays using synthetic vesicles and eukaryotic cells. Confirming purity of IgG is important in analysis as it reduces confounding effects from several other components found in whole antisera. Small-scale test purifications were utilized before a large-scale method was selected for purifying the IgG from the antisera. At this stage, the pre-immunization antisera samples were also purified to further evaluate non-specific binding and ability to block peptide activity. Altogether, this allows us to determine which method yields the highest purity IgG that can bind and recognize the majority of delta-peptide variants, and finally block permeabilizing activity of the delta-peptide against synthetic membranes and eukaryotic cells. Although both
rabbits produced IgG in response to the antigen used to immunize them, it is also likely that there are differential binding kinetics of the IgG based on the ability of the antibody to bind delta-peptide mutants as shown in the previous chapter. This is likely due to how the antigen was processed in each animal that was immunized. As such, it will also be important to determine which antibody from which animal will be used for in vivo studies. This section will lay the foundational groundwork for the preclinical evaluation of the custom IgG developed against the EBOV delta-peptides.

**Materials and Methods**

*Coupling azidoE23ox to DBCO-beads in a resin to develop a novel affinity purification strategy*

Although bead resins are commercially available for several types of affinity purification (Protein A/G/L and KLH), no such beads exist for specifically E23ox itself. Thus, they must be custom-engineered by our laboratory for usage in purification. Click chemistry was once again used in this portion of the study. By using azidoE23ox, we were able to purchase commercially available DBCO-linked agarose beads and chemically react them with our azidoE23ox peptide to create custom beads which had a modified E23ox coupled to them. This reaction was carried out over the course of a week and peptide was added in excess compared to the concentration of beads to drive the reaction forward. All reactions were carried out with PBS as the majority buffer. The click chemistry reaction was tracked by detecting free azidoE23oox in the reaction mixture supernatant and was confirmed by HPLC; success of the strategy was determined through ELISAs described in the following sections.

*Using small-scale tests to evaluate several purification strategies*

Since the limiting reagent in this study is the antisera (less than 400 mLs total from both rabbits combined), it is necessary to use small-scale tests in order to optimize the large-scale purification
strategy. As such, five different strategies were tested for the pre-immunization and post-immunization antisera; a table describing these strategies can be found in Figure 4-1. Here, affinity based on the Fc fragment (Protein A-linked agarose beads) and Fab fragment (E23ox/KLH-linked beads) were both employed to determine the optimal purification strategy (Figure 4-2). However, in order to have enough IgG to evaluate the individual strategy, 5 mLs of antisera per sample was used in each small-scale preparation. The first strategy involves no affinity purification, rather a molecular weight cutoff (MWCO) Millipore spin concentrator at a 100 kDa cutoff was used to spin down and concentrate pure antisera. This cutoff was used was used since native IgG is 150 kDa. The second strategy involved purification of the pre-immunization antisera using Protein A purification along with a spin concentration with a MWCO of 50 kDa. This strategy was chosen as there should be no antibodies against KLH or E23ox, so the whole IgG fraction was pulled down. The third strategy involved purification of the post-immunization antisera using Protein A purification along with a spin concentration with a MWCO of 100 kDa. This strategy was employed in purifying the IgG from the antisera derived from both rabbits. The fourth strategy involved a double affinity purification of the post-immunization antisera using Protein A as the primary affinity purification to pull down all IgG available and E23ox-linked beads as the secondary affinity purification step to pull down only E23ox-specific IgG. The sample is then spin concentrated using a MWCO of 50 kDa. The antisera from only one rabbit was used for this strategy as the DBCO-beads were cost-prohibitive and modified delta-peptide reagents were limited. The fifth and final strategy involved a double affinity purification of the post-immunization antisera using Protein A as the primary affinity to pull down all IgG available and KLH-linked beads as the secondary affinity purification step to remove all KLH-specific IgG from the large IgG pool. The sample is then spin concentrated using a MWCO of 50kDa. The antisera from both rabbits was used for this strategy since KLH-linked beads are commercially available and affordable. All IgG samples
derived from these strategies were then used in a BCA assay to determine total protein content and concentration. All purification steps were carried out using DPBS as the buffer.

**Evaluation of all small-scale purification strategies to select a large-scale strategy**

Once all small-scale strategies were complete, the IgG samples were tested for purity using SDS-PAGE gels and for reactivity using Western blotting and ELISAs. BCA assays were conducted after each purification to determine total protein content and concentration. Each purified IgG sample was first tested as the primary antibody for a Western blot at the same concentration using an HRP-linked secondary antibody for chemiluminescent imaging. Following these results, an indirect ELISA was conducted in which E23ox was coated onto a 96-well plate at a concentration of 1 µg/mL and the antibody samples derived from each purification strategy were serially diluted and tested for immunoreactivity using a BD Bioscience TMB Development Kit to visualize the results. Here, for many samples, only an n=1 or n=2 could be achieved due to the limited amount of antibody purified and concentrated. The large-scale strategy chosen was single affinity using Protein A purification only with spin concentration and buffer exchange with a 100 kDa MWCO Millipore filter.

**Using ELISAs to determine variant binding ability and sensitivity**

Once the antibody was purified in large amounts, two more indirect ELISAs were conducted. One ELISA tested variant binding ability by testing several delta-peptides (E14ox, E15ox, E17ox, E18n, E23red, E23ox, E23scr(ox), and E40ox). The peptides were coated at a concentration of 1 µg/mL on a 96-well plate and the purified IgG was diluted to a concentration of 25 µM and serially diluted in a 1:4 ratio down the plate. The previously mentioned TMB kit was used to develop the plate for visualization at 450 nm read by a plate reader. This assay was conducted at n=3. Following this assay, another set of ELISAs were conducted only using E23ox as the coating antigen for the 96-well plate starting again at a concentration of 1 µg/mL, but then serially diluted across the plate
The purified IgG was then diluted to a concentration of 25 µM and serially diluted in a 1:4 ratio down the other axis of the plate, creating a checkerboard. The TMB kit was used to develop these ELISAs and the plates were read by plate reader at n=3.

**Small molecule dye leakage from synthetic vesicles to determine preliminary a therapeutic index**

In order to begin profiling the therapeutic potential of a polyclonal antibody population against the delta-peptide, it is first necessary to determine the molar ratio of delta-peptide:IgG necessary to decrease or inhibit viroporin permeabilizing activity against synthetic vesicles. Here, a system in which POPC and POPG phospholipids are extruded into vesicles and used to entrap two small molecules: a fluorophore (ANTS) and its quencher (DPX). The ANTS fluorescence is quenched by DPX within the vesicle, but when the peptide perturbs the membrane and the small molecules can escape, ANTS fluoresces. The concentration of vesicles is determined using the Stewart assay. Peptide can then be added to the vesicles and fluorescence can be tracked by using a plate reader. The sensitivity of vesicle membranes can be used to our advantage by using small concentrations of peptide and IgG. Here, a constant concentration of 1 mM PC:PG ANTS/DPX-entrapped vesicles were used and peptide was serially diluted from a starting concentration of 100 µM while the IgG is at a fixed concentration of 25 µM. In this manner, we are able to test ratios that are both in favor of the peptide and in favor of the IgG in one plate. Ultimately, the determination of the ratio of IgG:peptide which reduces fluorescent signals produced by ANTS leakage will be used to optimize the blocking activity of the IgG in the context of eukaryotic cells.

**Using CHO cells to determine blocking against permeabilizing activity of the delta-peptides**

Once the ratio of IgG:delta-peptide necessary to reduce viroporin activity in synthetic vesicles was determined, CHO cells were grown in cell culture to test fractional SYTOX Green entry into cells as mentioned previously. The CHO cells were grown to 90-95% confluency in flasks before being split into 96-well plates for this assay. Here, instead of a serially diluted plate of peptides, only two
molar ratios of IgG:delta-peptide were tested (6:1 and 12:1) in order to conserve the amount of IgG used. Fractional SYTOX entry was then fluorescently tracked kinetically over the course of 2 hours. Both E23ox and E40ox were tested as well as the IgG purified from both rabbits. Both pre-immunization and post-immunization IgG is represented in these assays and MelP5 is used as a positive control pore-forming peptide. In this set of experiments, an n=6 was achieved for all experimental groups except for the pre-immunization antisera-derived IgG which was an n=1 or n=2 because of limited IgG stocks.

Results

**Coupling azidoE23ox to DBCO-beads in a resin to develop a novel affinity purification strategy**

Click chemistry was used to couple the azidoE23ox to the DBCO-beads to make a custom binding tool for another form of affinity purification. It is necessary to add the peptide in excess to the beads in order to ensure that the reaction is driven forward, and the maximum amount of peptide is coupled to the beads to increase their efficiency in affinity purification. The peptide was added in molar excess to the DBCO beads and was allowed to react over the course of one week. The reaction was tracked by HPLC, by extracting a known volume from the supernatant after letting the beads settle. The peak can be seen decreasing in height over time as the reaction occurs over the course of a week (Figure 4-3). This indicates that the peptides have bound to the DBCO-beads as less detectable free peptide was observed in the reaction supernatant. Fortunately, since the reacted peptide was now covalently bead-coupled, several bead washes were used to get rid of excess unreacted azidoE23ox peptide.

**Evaluation of all small-scale purification strategies to select a large-scale strategy**
Since the recovery of IgG from antisera results in an overall loss of protein content, more antisera must be used to obtain a higher concentration of IgG. Here, several strategies were tested via affinity purification in order to assess the optimal method for large-scale purification. The small amount of IgG obtained from the small-scale test purifications allowed for one Western blot and one ELISA for each strategy. The SDS-Page gels show the IgG derived from each strategy and run on the gel in 1:10, 1:100, and 1:1000 dilutions (Figure 4-4). All strategies used resulted in IgG fractions which were pure and resulted in bands around the 150 kDa band, which is the molecular weight of IgG, although it appears that there are trace amounts of other smaller proteins. The Western blots show that all appropriate IgG (derived from post-immunization antisera) can bind and recognize E23ox and E40ox (Figure 4-5). However, the ELISA reveals more details about the binding ability of the IgG derived from each strategy tested. The pre-immunization IgG is unable to detect E23ox, but all post-immunization is able to detect E23ox. Interestingly, it appears that using a singular affinity (Protein A only) purification strategy instead of a double affinity (Protein A + E23ox/KLH) results in IgG which is able to most sensitively detect the peptide (Figure 4-6).

**Using ELISAs to determine variant binding ability and sensitivity**

Once the large-scale strategy was chosen, it was also important to determine the variant binding ability and sensitivity of the IgG from rabbit 22017 in detecting the delta-peptides. When testing the EBOV delta-peptide variants, the IgG derived using the singular affinity protocol was able to detect E14ox, E15ox, E17ox, E23ox, E23red, and E40ox (Figure 4-7). However, the IgG was not able to detect E18n beyond background levels and the binding profile for E23scr(ox) appears to diminish with higher levels of IgG, but there was still a notable signal for E23scr(ox). Interestingly, when testing the delta-peptide coating the 96-well plates in a serially diluted manner in order to further test the sensitivity of the antibodies, we find that the IgG is able to detect the peptide even at the lowest concentration of peptide used to coat the plate and could likely detect peptide at even lower concentrations, which may have implications in the design of an EBOV diagnostic test based
on this antibody (Figure 4-8). This suggests that the polyclonal IgG pool derived from the post-immunization antisera using the selected strategy for large scale purification can detect several EBOV delta-peptide variants and is able to do so in a sensitive manner.

**Small molecule dye leakage from synthetic vesicles to determine preliminary a therapeutic index**

Once it was established that the IgG derived from large-scale purification is able to sensitively bind and detect several delta-peptide variants, it was necessary to determine the potential for the antibody to act as an inhibitor of the permeabilizing viroporin activity of the peptides. As such, PC-PG vesicles which had ANTS/DPX entrapped were used to conduct a small molecule dye leakage assay. Here, the vesicles were incubated with either peptide or peptide which was pre-incubated with the IgG and was read on a plate reader (Figure 4-9A). It is possible to observe that fluorescence is reduced in the E23ox + antibody (Ab) sample as well as the E40ox + Ab compared to E23ox and E40ox alone, respectively. The data suggests that a 5:1 molar ratio of IgG:E23ox pre-incubated for 1 hour prior to addition to the vesicles results in a 70% reduction of permeabilization of vesicles. The IgG pre-incubation also results in a reduction of E40ox activity, but this reduction is not as pronounced as the effects against E23ox. Overall, this suggests that treatment of the delta-peptide with the custom IgG may have a protective effect against synthetic vesicles. It is also important to note that IgG seems to cause about 20% leakage by themselves due to the sensitivity of the PC-PG vesicles (Figure 4-9B).

**Using CHO cells to determine blocking against permeabilizing activity of the delta-peptides**

Once a concentration and ratio of IgG:delta-peptide was determined to have a protective effect for permeabilization in synthetic vesicles, CHO cells were used to further the preclinical evaluation for the polyclonal IgG purified. In this section, it is possible to observe that fractional SYTOX Green entry, although slowed by the IgG, is not able to stop MelP5 from permeabilizing cells even at a
ratio of 24:1 (IgG:MelP5) (Figure 4-10A). The fractional SYTOX Green entry seems to decrease over time, however, this is a known phenomenon as the cells become so damaged from the MelP5 treatment, that they begin to de-adhere from the bottom of the wells, making the fluorescent focal point hard to detect for the plate reader. When testing the post-immunization IgG against E23ox, the IgG from both rabbits (22017 & 22018) are able to decrease the fractional SYTOX Green entry into CHO cells at both the 6:1 and 12:1 (IgG:E23ox) molar ratios (Figure 4-10 B & C). When testing the post-immunization IgG against E40ox, similar to the synthetic vesicle study, the IgG from both rabbits (22017 & 22018) are able to decrease the fractional SYTOX Green entry into CHO cells at both the 6:1 and 12:1 (IgG:E40ox) molar ratios, but not to the same degree as E23ox (Figure 4-10 D & E). Although the pre-immunization IgG is able to delay the effects, the permeabilizing effect of E23ox and E40ox is still observable at the later time points of the kinetic run. When looking at the ability of the IgG from each rabbit (22017 and 22018) to reduce fractional SYTOX Green entry into CHO cells, we find that the IgG from rabbit 22017 is able to inhibit the EBOV-delta-peptide viroporin activity to a greater extent compared to rabbit 22018 (Figure 4-11). This also further confirms that the post-immunization antisera from both rabbits contain IgG specific to the delta-peptides that the pre-immunization antisera don’t contain, and the post-immunization IgG is able to bind and neutralize the activity of the delta-peptides in a therapeutic context.

Discussion

Although several strategies for IgG purification were tested in this section, it appears that a singular affinity purification using Protein A-linked agarose beads resulted in an IgG pool which had the highest titer of antibodies that could bind the EBOV delta-peptides. A study by Sheng & Kong has also shown that IgG can bind so strongly to the native antigen, that it is difficult to release the antibody from the antigen, without also resulting in protein denaturing\(^{145}\). Ultimately, Sheng & Kong suggest the use of lower affinity antigens to avoid denaturing of the protein antigen during
elution; in this case, the technique was reversed and instead of IgG-linked beads, the previously described E23ox-linked agarose beads were used for immunoaffinity purification. However, the principle likely remains the same, that it is difficult to elute the high affinity IgG off of the E23ox-beads in the column during the purification process without using an elution buffer that denatures the IgG. In this case, it appears that the IgG pool is stronger when conducting purification from antisera by using affinity against the Fc fragment rather than the Fab fragment of the IgG molecule. This resulted in a polyclonal pool of IgG, which is expected when using KLH-based immunization strategies and Protein A-based purification strategies\textsuperscript{146,147}.

However, once the large-scale purification process was completed, the polyclonal IgG pool was shown to bind several delta-peptides and related variants. As the delta-peptide length is reduced by N-terminal truncation from 40 residues to 14 residues, the purified post-immunization IgG is still able to recognize the truncated forms of the delta-peptide, further indicating a polyclonal IgG pool as it can potentially recognize multiple epitopes. Polyclonal IgG can be effectively used as therapeutics and as diagnostic tools\textsuperscript{138,141}. Fortunately, in this section, tests with synthetic vesicles and eukaryotic cells have demonstrated the capacity of the IgG to not only bind the delta-peptides, but also to neutralize their activity. Interestingly, one of the rabbits (22017) generated antibodies which are able to reduce the delta-peptide viroporin activity to a greater extent than the other rabbit (22018). However, this is a common occurrence and relates to the manner of processing and presentation of the antigen in order to mount an immune response against the foreign antigen\textsuperscript{148,149}.

Moreover, these results support the novel conjugation strategy described in the previous section. The resulting IgG from the immunizations with the antigen that we engineered has the ability to bind and reduce viroporin activity of the EBOV delta-peptides. This represents a novel druggable target in the field of EVD therapeutics, an ongoing effort in response to the most current EBOV outbreak (2018-2020). At this stage, the EBOV delta-peptides have been characterized as enterotoxic viroporins \textit{in vivo}, suggesting that the delta-peptide variants represent a previously
undescribed, small set of viral virulence factors within the Filoviridae family. This possibility is further supported by the genetically conserved nature of the peptide and how it appears to be one of the most abundant products of the GP gene. Furthermore, efforts to turn the delta-peptide into an immunogenic target have been successful and the resulting IgG produced against the engineered antigen can reduce the viroporin effects against synthetic vesicles and eukaryotic cells once an appropriate purification strategy was chosen. These results represent a significant step forward in understanding the role of the delta-peptide in EBOV pathogenesis and EVD progression.
Figures

Figure 4-1

<table>
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<th>Antisera details</th>
<th>Strategy 1</th>
<th>Strategy 2</th>
<th>Strategy 3</th>
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</tbody>
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Figure 4-1: Small-scale affinity purification strategies used to pull down IgG from whole antisera

Here, six different strategies used to purify the IgG fraction of the antisera were tested for their efficiency and ability to pull delta-peptide specific IgG. Each strategy ranges from 0 to 2 types of affinity purification coupled with spin concentration using the MWCO indicated in the table. The antisera details indicate which bleed # was used for that specific test purification (ie. B18 is bleed #18). In strategy 4, ‘various’ is listed under the antisera details; this indicates a mixed antisera pool, specifically a combination of bleed 18 and the final bleed.
Figure 4-2: Optimization of affinity purification for IgG from whole antisera
The top section of the panel represents the principles behind the immunization and bleed process as well as the collection of polyclonal IgG and IgM in the antisera. The lower panels represent the two different types of affinity purification tested at this stage. IgG can be purified by the Fc fragment (Protein A) or by the Fab fragment (E23ox/KLH) in the small-scale test purifications. Not pictured here are the Fab affinity purification beads which were coupled to KLH, though this figure is more designed to illustrate the two different types of affinity purification being used.
Figure 4.3

Reaction Mixture Supernatant HPLC:
azidoE23ox

Time: start

Time: 48 hours

Time: 1 week
Figure 4-3: Tracking the coupling reaction between azidoE23ox and DBCO agarose beads by HPLC

Here, the reaction mixture was sampled at various time points to track the reaction between DBCO-linked agarose beads and azidoE23ox by using click chemistry. The sample of reaction supernatant was run on HPLC and the peaks for azidoE23ox (in red arrows) were observed over time. The reduction in peak height indicates that the reaction occurred, and that the peptide was coupled to the beads for custom affinity purification. Within 48 hours, the amount of detectable free peptide within the reaction mixture supernatant had reduced by half of what was detected at the start of the reaction. By one week, this peak was further reduced slightly, but has begun to plateau. This indicates that most available DBCO-linked beads had been coupled to the peptide within 48 hours.
Figure 4-4: Testing the purity of the IgG samples obtained from small-scale purification by SDS-Page
Once each small-scale strategy was complete, each IgG sample was run on SDS Page gels to rudimentarily determine IgG purity of the samples. Each sample of IgG was run at a 1:10, 1:100, and 1:1000 dilution from the stock and a high molecular weight ladder was run on all gels to determine where the 150 kDa IgG molecules would be found. Here, we can observe that most strategies resulted in fairly pure IgG samples with minimal banding outside of the 150 kDa band. However, it appears that there is an abundance of other proteins in the Antisera only + 100 kDa MWCO sample, indicating that at least one type of affinity purification is necessary for higher purity IgG samples.
After purity of the IgG samples were confirmed, Western blotting using E23ox and E40ox were used to determine binding ability of the IgG pulled down from the different affinity purification strategies. As expected, the IgG pulled down from the pre-immunization antisera did not bind E23ox nor E40ox. However, both strategies which employed double affinity purification (Protein A-linked beads + E23ox/KLH-linked beads) resulted in IgG which were able to bind and recognize both E23ox and E40ox.
Figure 4-6: ELISA to quantify binding capacity of IgG derived from each purification strategy

By using an ELISA in addition to the Western blots, we are able to make quantitative observations about the binding capacity with respect to sensitivity regarding the IgG purification strategies. As expected, the pre-immunization IgG results in little to no signal. However, all other small-scale purification strategies show varying degrees of reactivity to E23ox, which was used to coat the plate. Here, we find that the single affinity purification strategy (Protein A only) resulted in the most sensitive IgG samples compared to the double affinity purification strategies (Protein A + E23ox/KLH). Accordingly, the single affinity Protein A strategy was adapted for large scale use in order to purify a large stock of IgG for functional assays.
Figure 4-7: ELISA quantification of delta-peptide variant binding ability by purified IgG

Testing several variants of the EBOV delta-peptides by ELISA revealed that the IgG derived from rabbit 22017 was able to detect varying lengths of the delta-peptide (E14ox, E15ox, E17ox, E23ox, and E40ox) very sensitively. E18n (in orange) was not detected however, as it was not part of the original engineered antigen and this was an expected result. However, there appears to be an interesting signature regarding E23scr(ox), which is the motif scrambled variant of E23ox where large sections of amino acids were swapped around the sequence of E23ox. This potentially leaves recognizable epitopes intact and may be the cause of the binding and recognition of the peptide. The signal seems to diminish more with higher concentrations of antibody, but a signal is still present.
Figure 4-8: Testing the sensitivity of the IgG by ELISA in detecting lower concentrations of E23ox

Evaluation of concentration-dependent recognition of E23ox by the IgG revealed that the peptide may be recognized at the tested higher concentration of 2 µg/mL, which is actually multiple orders of magnitude lower than what has been reported for the delta-peptide *in vitro* when cells are infected with EBOV\textsuperscript{66}. The lowest concentration tested in this series was 0.0313 µg/mL, which was also appreciably detected by the antibody. This suggests that the concentration of E23ox could be even lower than that and a signal may still be generated, further validating the high sensitivity of the IgG in detecting delta-peptides.
Figure 4-9

A

![Graph showing ANTS/DPX Leakage against P:L ratio for different samples. The graph includes lines for MELP5, EX23, EX40, MELP5 Ab, EX23 Ab, and Ex40 Ab.]
To assess the ability of the IgG as a potential therapeutic agent, evidence of blocking activity against the EBOV delta-peptide must be generated. Here, using a synthetic vesicle system and using ANTS/DPX-entrapped PC-PG vesicles, a leakage assay was conducted to determine neutralizing ability of the IgG. Fluorescence is read by the plate reader and the x-axis indicates the peptide to lipid (P:L) ratio. The top trace (A) uses the antibody-treated vesicles as the baseline for all leakage and it is possible to observe a right shift from the red trace (E23ox only) to the pink trace (E23ox + IgG) indicating that more peptide is necessary to cause the same amount of leakage. This effect also occurs with E40ox, but not to the same degree as E23ox. Interestingly, it appears to mildly

Figure 4-9: Preliminary preclinical evaluation of the IgG as a potential therapeutic agent
enhance the permeabilizing activity of MelP5. However, this result is acceptable since MelP5 binding did not decrease, as that would indicate non-specific binding and reduction of permeabilizing activity. The bottom trace (B) shows the same trace, but with intact vesicles as the baseline for all leakage. Here, we are able to observe that the IgG actually causes ~25% leakage of vesicles by itself, suggesting that the vesicles are quite sensitive to even slight membrane perturbations. Overall, this experiment reveals that a 5:1 molar ratio of IgG:E23ox pre-incubated for 1 hour prior to addition to the vesicles results in a 70% reduction of permeabilization of vesicles. This indicates therapeutic potential of the IgG.
Figure 4-10

A

12.5 μM MelP5
$50 \mu M \text{E23ox}$

Fractional SYTOX Green Entry

Minutes

Legend:
- Blue diamond: Blank (300 µM 22017 IgG)
- Green square: Blank (300 µM 22018 IgG)
- Blue circle: 50 µM E23ox
- Red triangle: 50 µM E23ox + 300 µM Pre-Immune IgG
- White diamond: 50 µM E23ox + 300 µM 22017 IgG
- Green square: 50 µM E23ox + 300 µM 22018 IgG
Figure 4-10: Assessment of IgG therapeutic index in the context of SYTOX Green entry into CHO cells

(A) An unrelated membrane-permeabilizing peptide, MelP5, was chosen to test the different antibody preparations by measuring fractional SYTOX Green entry into CHO cells which were seeded into 96-well plates and read on a plate reader. There are 3 antibody preparations being tested in each of these panels: pre-immunization IgG, rabbit 22017 post-immunization IgG, and rabbit 22018 post-immunization IgG. This is done to assess the inhibition of permeabilizing activity by the peptides. Here, we find that although the permeabilizing effect is delayed, permeabilization of cells by MelP5 still occurs even at a 24:1 molar ratio of IgG:MelP5. The reduction of increasing fractional SYTOX Green entry followed by decreasing fractional SYTOX Green entry over time
is due to the de-adherence of the CHO cells from the bottom of the well in response to MelP5 treatment. (B) When testing 50 µM E23ox, we find potent permeabilizing activity against CHO cells as indicated by the ‘E23ox only’ trace and the pre-immunization IgG did not greatly hinder this permeabilizing activity. However, an overnight incubation of IgG with peptide at a 6:1 molar ratio of IgG:E23ox appears to neutralize the permeabilizing activity almost completely. It appears that IgG from rabbit 22017 is more fully able to inhibit the E23ox peptide compared to the IgG from rabbit 22018. Additionally, the IgG alone treatment at a concentration of 350 µM seems to be well tolerated by the CHO cells. (C) This effect is further exemplified when testing 25 µM E23ox, as this would represent a 12:1 molar ratio of IgG:E23ox. This ratio was also able to ablate almost all activity completely with IgG from both rabbits (22017 & 22018). (D) When testing 50 µM E40ox, we find potent permeabilizing activity against CHO cells as indicated by the ‘E40ox only’ trace and the pre-immunization IgG did not greatly hinder this permeabilizing activity. However, at a molar ratio of 6:1 (IgG:E40ox), we find that both 22017 IgG and 22018 IgG are able to decrease fractional SYTOX Green entry by E40ox, but not to the same extent as E23ox. (E) Interestingly, when testing 25 µM E40ox at the 12:1 molar ratio of IgG:E40ox, we find that almost all permeabilizing activity is inhibited. This indicates that even though E40ox wasn’t the original antigen, the IgG is still able to exert protective effects at a slightly higher concentration.
Figure 4-11

A

Rabbit 22017

- Blank (300 μM 22017 IgG)
- 50 μM E40ox
- 50 μM E23ox
- 50 μM E40ox + 300 μM 22017 IgG
- 50 μM E23ox + 300 μM 22017 IgG
Figure 4-11: Consideration of the source of IgG and exerted protective effects against delta-peptides

(A) Here, we consider the IgG only from rabbit 22017 and the protective effect of the IgG only in the 6:1 molar ratio of IgG:delta-peptide. We find that E23ox and E40ox at 50 µM are able to exert potent permeabilizing activity against CHO cells and that fractional SYTOX Green entry is decreased by ~90% with respect to E23ox and decreased by ~70% with respect to E40ox. This indicates broadly protective effect of the IgG in the context of mammalian cells. (B) In this panel, only the IgG from rabbit 22018 is considered and again, only at the 6:1 molar ratio of IgG:delta-peptide. The IgG from rabbit 22018 is able to decrease fractional SYTOX Green entry by ~85%
with respect to E23ox and by ~40% with respect to E40ox, also indicating protective effects. However, it appears that 22018 IgG is not as protective as 22017 IgG.

CHAPTER 5: Assessment of a novel polyclonal IgG therapeutic strategy against the delta-peptide \textit{in vivo} and considerations for usage as a diagnostic tool

Introduction

Currently, the landscape of EBOV of therapeutic and diagnostic tools is still evolving especially as new technologies are developed. The current preferred method of detection is a PCR-based assay\textsuperscript{150,151}. Although the PCR-based diagnostics are sensitive, this method requires EBOV to become detectable in the blood after the onset of symptoms. This can take 3-10 days to reach sufficient detectable levels\textsuperscript{152}. Alternatively, there are diagnostic approaches being developed which are antibody-based and can serve as a secondary confirmatory diagnostic test supplementing the PCR approach\textsuperscript{153}. However, since the EBOV sGP and delta-peptide seem to be the majority product made from the GP gene during active pathogenesis and viral replication, the custom antibody against delta-peptide may be useful in early detection\textsuperscript{64,65}. This may be especially true since gastrointestinal pathology is seen early in the onset of symptoms post-EBOV infection. Accordingly, there is a need to improve diagnostic ability even further as earlier interventions of therapeutics improve recovery outcomes with respect to EBOV and other viruses\textsuperscript{153}.

There are also therapies being developed and evaluated, several of which rely on antibody-based inhibition of EBOV\textsuperscript{56,99}. In the previous chapter, the preliminary preclinical evaluation of the delta-peptide antibody from rabbit 22017 revealed that a 6:1 molar ratio of IgG:delta-peptide preincubated overnight was able to neutralize the majority of permeabilizing viroporin activity against CHO cells. In this chapter, the preclinical evaluation of the delta-peptide antibody will be
extended by returning to the murine diarrheal pathology model and observing the mice for protective effects provided by the antibody against gastrointestinal distress induced by the EBOV delta-peptides. The ability to block diarrheal pathology by the delta-peptide would effectively demonstrate that the EBOV delta-peptides are not only enterotoxic viroporins, but they are also druggable targets \textit{in vivo}. Although it is very unlikely that the delta-peptides are the sole cause of gastrointestinal distress seen in EBOV patients, this would represent a novel therapeutic target for EBOV and could potentially be used to manage a portion of the gastrointestinal pathology symptoms commonly seen in EVD. Additionally, these tests represent the downstream applications and utility of the antigen engineering strategy described in Chapter 3. Although the EBOV delta-peptides are the focal point of this study, the principles behind the peptide conjugation strategy to the carrier protein without the use of lysine chemistry on the part of the antigen may be applied to other haptens which are otherwise difficult to use as immunogens.

\textbf{Materials and Methods}

\textit{Development of a modified ELISA to determine diagnostic ability of the custom IgG}

In order to crudely test the sensitivity of the antibody in recognizing the antigens (EBOV delta-peptides) in the context of human serum to mimic patient samples, the peptides were diluted with DPBS (20\% Human Type AB serum). The ELISA tested the ability of the antibody to recognize several delta-peptides (E14ox, E15ox, E17ox, E18n, E23red, E23ox, E23scr(ox), and E40ox) in the context of human serum. The serum-diluted peptides were coated at a concentration of 2 \(\mu\)g/mL onto a 96-well plate and the purified IgG was diluted to a concentration of 25 \(\mu\)M and serially diluted in a 1:4 ratio down the plate. The previously mentioned TMB kit was used to develop the plate for visualization at 450 nm read by a plate reader. This assay was conducted at \(n=3\).

\textit{Returning to the murine diarrheal pathology model to assess protective effects afforded by the IgG}
To fully evaluate the protective capacity of the antibody against the EBOV delta-peptides *in vivo*, the previously described murine diarrheal pathology model was revisited in a small pilot study. Using the CHO cells, a 6:1 molar ratio of IgG:delta-peptide afforded protective effects against fractional SYTOX Green entry into the cells. For this section, a 7:1 molar ratio of IgG:delta-peptide was preincubated overnight prior to injection into the surgically ligated ileal loop. The concentration of E23ox and E40ox used was 50 µM, which has previously demonstrated potent ability to cause diarrheal syndrome (Figure 2-4). A singular time point of 9 hours was also utilized, as this was previously demonstrated to be one of the peak time points for diarrheal syndrome and ileal loop fluid accumulation after introduction of the delta-peptides to the loop. The IgG from rabbit 22017 was used at a concentration of 350 µM for the preincubation stage and a total volume of 100 µL was injected as previously described. After 9 hours, the mouse was sacrificed, and the ileal loop was resected to determine the Loop Ratios.

**Results**

**Development of a modified ELISA to determine diagnostic ability of the custom IgG**

The ability to rapidly detect EBOV is crucial in early treatment and is associated with better health outcomes and transmission rates\(^1\). Here, an ELISA was conducted by doping a 20% human serum-DPBS mixture with 2 µg/mL of delta-peptides to act as a surrogate from a live patient sample and coating an ELISA plate with several delta-peptide variants. In Figure 5-1, we find that the 22017 IgG specific for delta-peptide has the ability to bind and recognize low concentrations of several variants of delta-peptide even in the context of human serum (E15ox, E17ox, E23ox, and E40ox). E18n and E14ox produce signals which are near background levels as indicated by a previously mentioned, unrelated peptide (ONEG). Although reduced from coated antigen with no human serum present, the antibody again demonstrates detection sensitivity. Currently, the PCR-based diagnostic techniques require a blood draw from the patient to test for detectable quantities of virus.
This experiment demonstrates that the same blood draw from a patient can potentially be spun down into just the serum fraction after the RBCs and WBCs have been pelleted and used to coat an ELISA plate and detect EBOV delta-peptides.

**Returning to the murine diarrheal pathology model to assess protective effects provided by the IgG**

Determination of the therapeutic capacity of the delta-peptide antibody is crucial in defining the potential benefits of targeting the EBOV delta-peptide during active infection. Here, we test the 22017 IgG against both E23ox and E40x in the murine diarrheal pathology model described in previous sections. In Figure 5-2, we consider all experimental groups and the ileal loop injection that they will receive. As this set of surgeries were conducted many months later than the initial study, we first compare the Loop ratios derived from the first round of surgeries to the last round of surgeries (Figure 5-3). Although the Loop Ratios are slightly different, we can observe that 50 µM E23ox still increases fluid accumulation past our designated threshold for diarrheal syndrome. In Figure 5-4, using a 7:1 molar ratio of IgG:delta-peptide preincubated overnight, we find that the IgG is able to neutralize diarrheal syndrome at the 9-hour time point at a statistically significant level with regard to E23ox. E40ox shows an elevated Loop Ratio, but it does not reach the threshold we determined. The E40ox + IgG treatment resulted in slightly lower Loop Ratios as well, but this reduction was not statistically significant. Furthermore, it appears that the IgG alone does not cause fluid accumulation and behaves similarly to the 0.025% AcOH negative control. Thus, the delta-peptide antibody can be used *in vivo* to decrease the enterotoxic effects of the EBOV delta-peptides without causing adverse effects at the IgG concentration tested.

**Discussion**

In this short chapter, we demonstrated important application details regarding the custom antibody we generated using our engineered antigen. Firstly, the 22017 IgG used in the ELISA to determine
diagnostic potential showed that the antibody can be used to sensitively detect several delta-peptide variants in a dilution of human serum. This demonstrates the potential use of this antibody as an alternative or confirmatory diagnostic test in relation to the PCR-based techniques. Further evaluation will have to be done using serum from actively infected patients to observe whether this technique would be useful in the context of an EBOV outbreak, but this represents a novel EBOV-specific target which can potentially be used for early diagnosis. Finally, over the last few chapters, we describe a full characterization of the action of the EBOV delta-peptides in the context of the small intestine and its potential role as an enterotoxic component of EVD pathology. After using alternative chemical engineering strategies in producing an antigen using this enterotoxic viroporin, we can purify and use the post-immunization IgG from two rabbits to block the permeabilizing viroporin activity of the delta-peptides in vitro using synthetic vesicles and eukaryotic cells. Furthermore, it appears that this IgG can also be used in vivo to block gastrointestinal distress induced by E23ox in the murine diarrheal pathology model without off-target effects, representing a novel target in EBOV therapeutic development and validating our antigen conjugation strategy.
Figures

Figure 5-1

20% Serum Detection ELISA (background subtracted)

Figure 5-1: A modified ELISA to detect EBOV delta-peptides in the context of human serum

Testing several variants of the EBOV delta-peptides diluted using 20% human serum in DPBS by ELISA revealed that the IgG derived from rabbit 22017 was still able to detect varying lengths of the delta-peptide (E15ox, E17ox, E23ox, and E40ox) sensitively. E14ox (in green) and E18n (in blue) were not detected, though E18n was not a part of the original antigen. Overall, the IgG from rabbit 22017 may be applicable in a diagnostic capacity based on the results of this ELISA.
Figure 5-2

<table>
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<th>350 µM IgG</th>
<th>50 µM E23ox</th>
<th>50 µM E40ox</th>
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Figure 5-2: Outline of the small in vivo pilot study evaluating the protective effects of the 22017 IgG

In a small pilot study testing the protective effects of the IgG against the EBOV delta-peptides, six experimental groups will be examined. Both E23ox and E40ox will be tested at 50 µM both with and without IgG at a 7:1 molar ratio of IgG:delta-peptide. Although 50 µM E23ox has been tested previously (resulting in increased Loop Ratios) at the 9-hour time point, 50 µM E40ox has not been tested in this fashion. Using antibody alone will also be tested to assess off-target effects of the IgG in the closed ileal loop.
Figure 5-3: A comparison of results from the murine diarrheal pathology model

This panel compares the Loop Ratios calculated for the surgeries from the mice in Chapter 2 to the most recent set of surgeries in this chapter. Here, we find that the controls behave similarly and the E23ox groups are both able to cause diarrheal syndrome beyond the threshold that was described earlier and indicated by the dotted line. Although the Loop Ratios of the E23ox groups are slightly different from each other, this is an expected result as *in vivo* work results in a lot more variability due to the differences in mice populations regardless of breed.
The aggregate data from Chapter 2 and this chapter were analyzed together in order to power the analysis further. Here, we find that there is a statistically significant difference between 50 µM E23ox and the 0.025% AcOH control as well as the 50 µM E23ox which was pre-incubated with the IgG overnight. This demonstrates that the IgG is able to block enterotoxic activity elicited by E23ox. We also find that the IgG alone did not cause gastrointestinal distress and although E40ox did not cause diarrheal pathology to the extent E23ox did, this effect is still lowered when
preincubated with antibody. For this plot, significance is designated as follows: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

**Future Directions**

There are several future directions worth exploring in this project, especially with the validation of the delta-peptide antibody described in the previous chapters. Immediate next steps would certainly involve conducting histopathological and RNASeq analysis as described in Chapter 2 regarding the resected ileal loops collected from the antibody *in vivo* trial. It would also certainly be worthwhile to expand the testing of protective effects provided by the IgG against other delta-peptides such as E14ox, E15ox, and E17ox in the context of synthetic vesicles, eukaryotic cells, and mice as previously done for E23ox and E40ox. It would also be prudent to optimize the preincubation time of delta-peptide with IgG to create a kinetic binding and inhibition profile. An *in vivo* model of vascular leakage using Evans blue may be used to assess the effects of the EBOV delta-peptides in the context of hemorrhagic symptomology\textsuperscript{154}. With respect to the antigen itself, consideration should be given to engineering another large pool of antigen using the previously described strategy with E40ox as the delta-peptide antigen of interest. Further consideration should be given to use this antigen as a potential vaccine and assessing the protective effects of circulating antibody against the EBOV delta-peptides. Since the antigen has been validated, it would also be prudent to follow protocols for the development of monoclonal antibodies and a hybridoma versus using intermittent immunizations and production bleeds to produce polyclonal antibodies. This would also help ease the purification process of the IgG as well. Since the IgG has now proven to be effective in blocking E23ox-induced diarrheal pathology *in vivo*, it would also be prudent to collaborate with BSL-4 level labs which are equipped to evaluate novel EBOV therapeutics to determine the efficacy of the antibody against live virus and potentially in a non-human primate
(NHP) model. Finally, further details regarding the enterotoxic mechanism in vivo may be elucidated by using the dye-labeled variant of the delta-peptide (E23ox-AF555) and tracking where the peptide goes in the murine diarrheal pathology model.

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Shantanu Guha was born on February 26, 1989 in Garland, TX, the first child of Tappan and Mita Guha. He received his primary education in Richardson, TX and in 2001, moved to McKinney, TX where he attended middle school and high school in the McKinney Independent School District. After graduating McKinney North High School in 2007, Shantanu attended Austin College in Sherman, TX, ultimately receiving a Bachelor’s (BA) degree in Biology in 2011. It was during this time at Austin College that he first developed an interest in research. Directly following college, Shantanu moved to New Orleans, LA to join Tulane University to earn a Master of Public Health (MPH) degree in Epidemiology, which was received in 2013. After a one-year hiatus from graduate school to gain laboratory and work experience, Shantanu started the Graduate Program in Biomedical Sciences at Tulane University in the fall of 2015 to earn his Ph.D.